# The Interaction of Phytoestrogens with Modified *Escherichia coli* as a Biosensor for Different Animal Species

**Guk Hee Steven Youn**

**Advisor: Dr. David W. Wood**

**The Ohio State University**

**William G. Lowrie Department of Chemical and Biomolecular Engineering**

**Autumn 2014**

# Table of Contents

# List of Figures

# List of Tables

## Abstract

The nuclear hormone receptors (NHRs) are important features of the cell, as they control activities of cell development, homeostasis, reproduction, and metabolism. Importantly, NHRs are also targets for naturally occurring and synthetic endocrine disrupting compounds. Any interference with their functioning has been connected to breast cancer, decreased fertility, osteoporosis, and other disorders in humans and other animals. We have constructed a very simple reporter system, or a biosensor, by modifying Escherichia coli by inserting an intein with the beta subtypes of the human estrogen receptor ligand-binding domain and thymidylate synthase reporter enzyme gene (D1210). With this modification, these *E. coli* cells can sense human estrogen and report their presence and interactions through changes in growth phenotype. The modified *E. coli* cells are referred to as biosensors and they have capabilities to distinguish estrogen agonistic growth and antagonistic suppression activities and report rough estimates of binding affinity. In this study, we will be conducting experiments to investigate the interactions of plant-derived estrogens, or phytoestrogens, and their estrogenic potency. In order to generate the data, biosensor cells are cultured in thymine-less media, and are introduced to test ligands. The biosensor growth phenotype allows the identification of which phytoestrogens will yield a similar potency to estrogen. The growth phenotype were determined by measuring the optical density (OD600) at wavelength of 600 nm. Our experiment results suggest that genistein and daidzein, which belongs to the category of isoflavones, yield similar potencies as estrogen. Comparisons between the tested phytoestrogens binding affinity were also studied and reported. While the estrogenic potency of industrial-originated estrogenic compounds is very limited, the findings of this study can hopefully reveal the estrogenic potency of phytoestrogens and also reveal what may trigger many of the biological responses that are evoked by the phytoestrogens and its ability to be a potential remedy for advanced endocrine related cancer.

## Introduction

The nuclear hormone receptors (NHRs) are a family of ligand-activated transcription factors present in animals. [1] NHRs control the expression of several genes as a response to the presence of hormones and hormone-like compounds. Some of the well-known members of this receptor superfamily are estrogen, androgen, thyroid hormone, progesterone receptors and more endocrine-related receptors. [4, 6] Nuclear hormone receptors is made up of very important classes of broad drug targets. [1] This is because NHRs have been linked to variety spectrums of diseases such as leukemia, breast cancer, endometrial cancer, prostate cancer, cardiovascular disease, osteoporosis, and inflammations. [2, 3] Thus, a discovery of novel compounds with ability to bind and modulate these receptors could lead to the development of valuable therapeutics against serious pathological conditions.[4, 6] The current method uses screenings of identifying hormonal compounds that exists as a form of *in vitro* competitive binding assays. Known as E-screen, this method observes the proliferation of human breast cancer cells in the presence of test compounds and evaluate their estrogenicity.[7] However, this method is not appropriate for the construction of high-throughput screening systems.[7] Furthermore, these approaches of utilizing animal cell-assays are generally complex, time-consuming, and expensive.[7] Another method that utilizes biosensor is known as Biacore T100. The Biacore T100 is a Surface Plasmon Resonance based biosensor for detecting, characterizing, and quantifying bimolecular interactions. The Biacore T100 employs a modified gold surface on which a ligand is immobilized. The sample analyses is then injected over this surface using an automated microfluidics system. Binding results in an increase in mass on the surface which is recorded as a change in instrument response over time (www.biocore.com). However, Biacore T100 is expensive and time consuming. Thus, a cheaper method must be constructed and utilized in order to accelerate the lead identification process and allowing new drugs to be discovered. This may be done with a construction of a simple *in vivo* assays using yeast or bacteria biosensor. [4, 6]

The ligand-binding domain (LBD) of the nuclear hormone receptors has shown to possess the ability to act as post-translational functional switches for a number of heterologous proteins when included as insertion fusions. [8,9] Furthermore, binding domains derived from the estrogen, androgen, and thyroid hormone receptors have been used to convey hormone-regulated activity to transcription factors, and other reporter protein such as dihydrofolate reductase (DHFR) and more.[4,8,9] This characteristics has been employed to develop many of the currently used *in vivo* screening strategies for endocrine modulators and also as a tool for deciphering different aspects of different hormone-regulated endocrine pathways in eukaryotes [4, 5]. However, the question of whether this property can be applied to the construction of

simple bacterial systems for high-throughput screening of test compounds remains unknown.

A recent publication in the journal of environmental toxicology (Gierach et al.) have reported a successful construction of a novel recombinant bacterial biosensor protein that includes a human, pig, and sole NHR LBD, which these LBDs are stabilized by an engineered mini-intein splicing domain and joined to a thymidylate synthase (TS) reporter enzyme. [4, 6] This bacterial biosensor undergoes conformational changes depending on either agonistic and antagonistic binding to the human, sole, or pig ERβ LBD. Subsequently, this binding leads to inducing a change in the TS reporter enzyme activity. Since TS acts as a catalyst of the methylation of deoxyuridylate to deoxythymidylate using 5, 10-methylenetetrahydrofolate (methylene-THF) as a cofactor and effects synthesis of thymine, the change in TS activity is shown by a change in growth phenotype of the *E. coli* cells, which can be quantified by growth measurements in a high-throughput format.[6, 11] TS is a crucial enzyme for the growth of biosensor cells because TS enzymes synthesize thymine in the biosensor cells, which is one of the four nucleobases in the nucleic acid of DNA. Without this TS gene (D1210), thymine will not be produced. This leads to termination of DNA replication, and subsequently eliminating the proliferation of the biosensor cells. This biosensor cell cultures are developed initially without the testing ligand during overnight/overday cultures; thus, we assume that the TS fusion proteins on the sensor module are inactive. When the modules are inactive, biosensor cells are not healthy and cannot proliferate. To overcome this problem, thymine is supplemented to cell cultures for the DNA replication and proliferation. Addition of thymine in cell cultures results in proliferation of cells that are healthy enough for biosensor tests. These bacterial biosensors also inform more information of the test compounds due to its ability to recognize the difference between agonist and antagonist. [5]

Another previously constructed estrogen binding sensor was constructed using *Saccharomyces cerevisiae*, which also provides guidelines for the construction of a simple screening assays [5]. This system reports the presence of active estrogen-alike compounds through growth phenotype on a selective medium [10]. This sensor is based on a chimeric fusion of the ligand-binding domain of the human estrogen receptor with temperature-sensitive mutants of dihydrofolate reductase (DHFR). Thus, DHFR-deficient yeast strains are able to grow at elevated temperatures only in presence of estrogen, which allows estrogen-alike compounds to be identified easily through phenotypical changes. This yeast assay was able to successfully detect estrogen analogues from a small library of test compounds. With advantages in speed and simplicity, this system offers the advantage that it can be adapted to accommodate different binding domains. Because of this, this yeast assay offers a general tool for reporting ligand-receptor interactions. However, one of the main shortcomings of this

yeast assay has been their inability to recognize the biological role of a test compound. For example, yeast assays has been shown to unable to discriminate reliably between agonistic and antagonistic effects of synthetic hormone mimics [5].

In this study, we describe the methods and results of bacterial sensors of nuclear hormone binding when induced by well-known phytoestrogens, estrogens, and other endocrine disrupters. The ligand-binding domain of the human estrogen β (ERβ), in combination with a stabilization and a solubilization domain, was fused to a TS enzyme. Expression of this fusion protein in TS-deficient *Escherichia coli* strains resulted in specific hormone-dependent phenotypes, which could be detected readily through changes in cell growth on selective thymine-free medium (-THY). [4, 6] On the previously reported study (Gierach et al.), several known hormone antagonists were to neutralize the effects of known agonist compounds on cell growth when added in combination, which indicates that this sensor is able to distinguish between antagonistic and agonistic activities [4]. Thus, it is expected that this bacterial biosensor will be an attractive alternative for performing the initial chemical compound library screening for the identification of compounds with potential therapeutic actions against serious diseases. [4, 6]

## Design and plasmid construction of a hormone-sensing ERβ biosensor protein

The design of the hormone-sensing biosensor protein is built on the principle of coupling the hormone-depending regulatory function of the LBD of a provided nuclear hormone receptor with the activity of the well-characterized TS enzyme. [4, 6] The activity of this biosensor cells can be monitored easily by measuring the growth phenotype in TS-knockout cells[11], and it has been used in several previously reported genetic selection systems.[12, 13] Because the amount of active TS required for growth increases proportionally with the incubation temperature, this bacterial biosensor protein system provides an accurate and quantitative measurement of TS enzyme activity by simply testing growth phenotypes over a range of temperatures [11-13] The expression of nuclear hormone-binding domains in *E. coli* is usually hindered by low solubility and poor stability; however, these problems can be avoided by the use of various gene fusions.[5, 14-18] Thus, stabilization and solubilization domains are included as parts of the constructed fusion.[5] This stabilization domain consists of a previously isolated mini splicing domain of the *Mycobacterium tuberculosis* RecA intein (Mtu intein).[5] Many derivative of this intein has been reported to fold properly and maintain activity when inserted into foreign protein hosts, [19] and can tolerate the genetic insertion of non-native short polypeptides [20] or entire folded protein domains. [21-24]

Previous works in Dr. Wood laboratory have indicated that the insertion of the ligand-binding domain of a nuclear hormone receptor into mini intein splicing domain allows the binding domain to adopt its correct fold and maintain its binding ability in *E. coli*. [4, 24] Additionally, including the maltose-binding protein tag (MBP) may increase the solubility and activity of the chimeric fusion. [25] Schematic representation of human, sole, and porcine biosensor genes are shown in figure 1 below. Notations are as follows: $P_{tac}$* = mutant tac promoter associated with hormone dependent phenotype; MBD = Maltose Binding Domain; N-Mtu = 5 first 110 residues of the Mycobacterium tuberculosis RecA intein; ERB = Estrogen Receptor B subtype ligand-binding domain; C-Mtu = last 58 residues of the Mtu RecA intein; TS = bacteriophage T4 thymidylate synthase enzyme. [4] Note that schematic representations of rat, cow, and zebra fish genes are not shown.

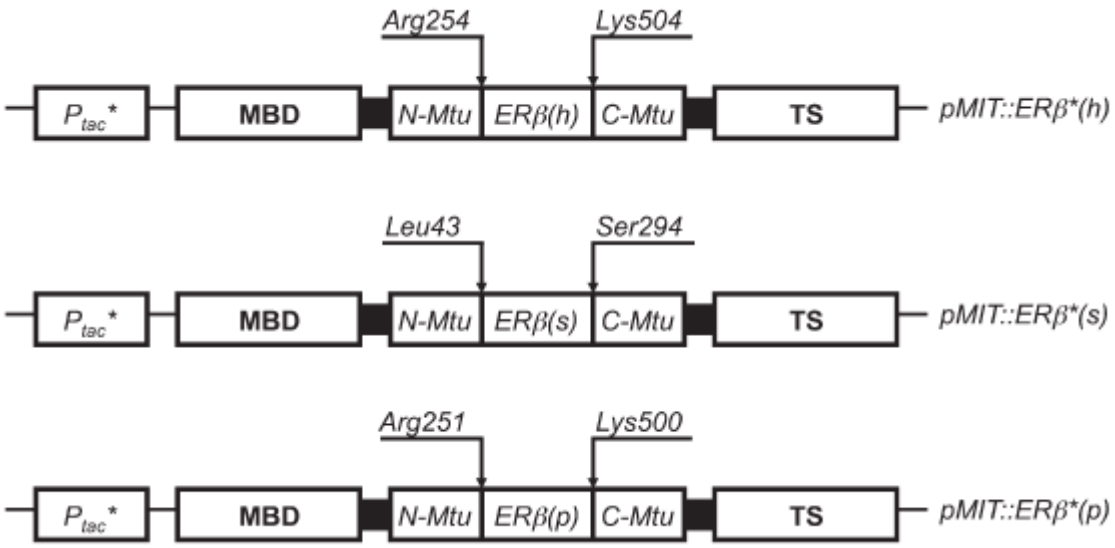



*Figure 2. Schematic representations of human, sole, and porcine biosensor genes.*

With these guidelines, endonuclease domain of the wild-type Mtu intein was replaced with estrogen receptors (ER) to form the initial chimeric fusion.[5] Splicing and N-terminal cleavage by the intein were suppressed by mutation of its initial amino acid from cysteine to alanine, while C-terminal cleavage was prevented by substituting the ultimate asparagine residue with alanine.[5, 12, 13] The established chimeric intein was fused to the N-terminus of the bacteriophage T4 *td* gene and the resulting fusion was cloned into plasmid pMal-c2 as a C-terminal fusion to the *E.coli* maltose binding domain.[5, 13] The T4 TS enzyme is known to complement TS-knockout bacterial cells; however, the td gene will not recombine with *E. coli* chromosomeal *thyA* gene.[13] The result of this construction is referred to as pMIT::ER (artificial *tac* promotor-MBP-intein-ts::estrogen receptor).[4,6] *E coli* D1210Δ*thyA* cells, which are deficient in native TS, were transformed with pMIT::ER and reported to unable to grow at all temperatures tested in a thymine-free medium, regardless of the presence of estrogen.[5]

Thus, the plasmid pMIT::ER was introduced into *E. coli* XL1-Red for the introduction of random mutations that might impart hormone sensitivity. This mutant was selected in E coli D1210ΔthyA for growth on thymine-free agar plates (TS+ phenotype) in the presence and absence of 17-β-estradiol (E2). After incubation, colony formation was observed to become estrogen-sensitive on plates incubated at 30°C. [5] It is shown that several of the mutants that exhibited TS+ phenotypes were sequenced and all were

found to contain a G to A nucleotide substitution in the lac operator region, 16 bases downstream of the TATAA motif of the *tac* promoter. [4, 6] No other mutations were observed and this suggests that that the observed positive phenotype is based on changes in expression level, most likely due to reduced affinity of the *lac* repressor for this recognition sequence. [5]

## Experimental Methods and Statistical Analysis of Results

Test compounds that were used in various experiments are 17-β-estradiol (E2), 5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one (Genistein), 7-Hydroxy-3-(4-hydroxyphenyl)chromen-4-one (Daidzein), 5,7-dihydroxy-3-(4-methoxyphenyl)chromen-4-one (Biochanin A), 5,7-Dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one (Apigenin), dimethyl sulfoxide (DMSO), and 4,4'-isopropylidenediphenol (BPA). These chemical were purchased from Sigma (St. Louis, MO, USA). The plasmas of all species to be tested were provided by Dr. David W. Wood (Ohio State University, Department of Chemical and Biomolecular Engineering). All other chemicals used for microbial cell culture were purchased from Fisher Scientific (Pittsburgh, PA, USA).

The design of the human, pig, sole, cow, rat, and zebra fish are referred to as pMIT::ERβ*(h), pMIT::ERβ*(p), pMIT::ERβ*(s), pMIT::ERβ*(c), pMIT::ERβ*(r), and pMIT::ERβ*(zf), respectively. These plasmids of different pMIT:: ERβ* have been transformed into the *E. coli* strain D1210ΔthyA::Kan$^{R}$ [F$^{-}$ *Δ(gpt-proA)62 leuB6 supE44 ara- 14 galK2 lacY1 Δ(mcrC-mrr) rpsL20 (Str$^{r}$) xyl-5 mtl-1 recA13 lacIq*] and plated cells on Luria-Bertani (LB) medium agar[4, 6]. The LB agar plate is supplemented with 100 µg/mL ampicillin and 50 µg/mL thymine. After all strains are plated, plates are transferred to an air incubator that is maintained at 37°C for colonies to grow. Figure 2 below illustrates plates after incubation at 37°C.

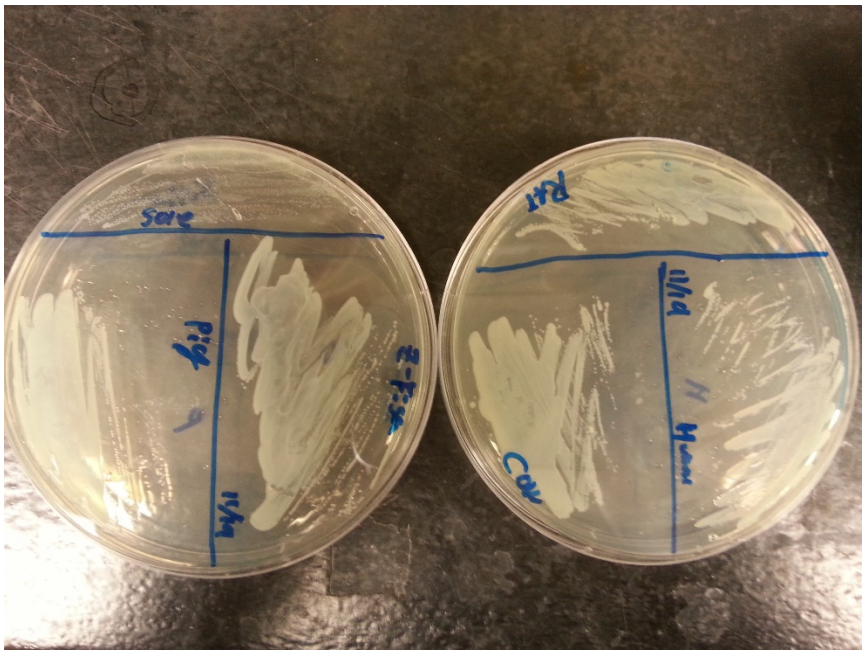



*Figure 2. A picture of plates after air incubation maintained at 37°C.*

Fresh colonies contacting the appropriate biosensor plasmid are introduced to a fresh 5 mL of LB liquid media in a tube that is supplemented with 100 µg/mL ampicillin

and 50 µg/mL thymine. After, tubes are transferred to water bath shaker that is maintained at 37°C and are grown over night for 15 hours. A picture of before and after of overnight procedure is shown in figure 3 below.

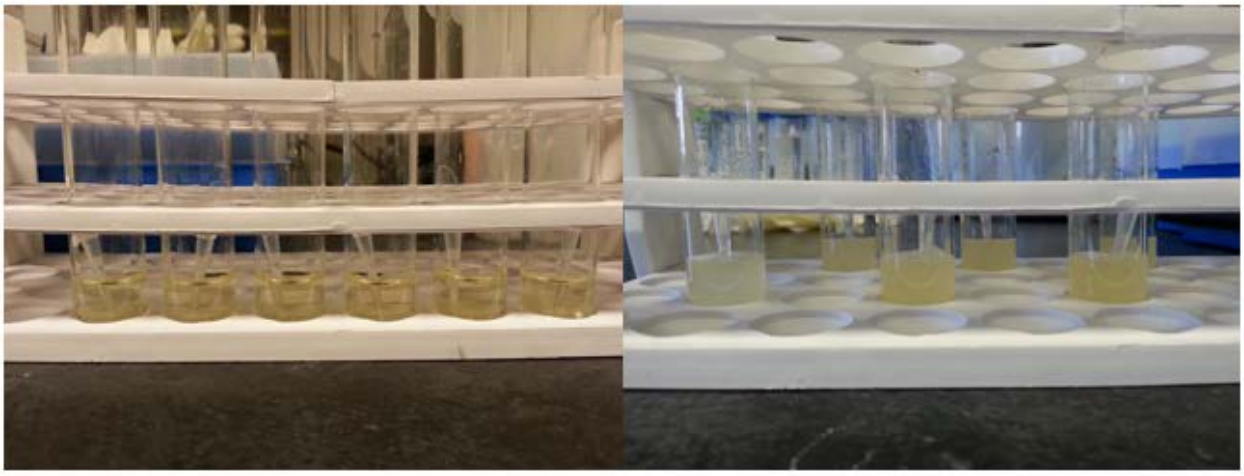


*Figure 3. Before and after picture of biosensor cells from left to right, respectively.*

Next, the overnight growth assays are transferred into fresh set of growth medium containing 5 mL of liquid LB, 125 µL of thymine, 5 µL of ampicillin and 50 µL of appropriate pMIT::ERβs cells. They were placed in the water bath shaker for about 4 hours or until they reach optical density of 1.3 to 1.5. These seed cultures were then diluted 1:200 in liquid thymine-free medium (-THY) (per liter: 10 ml of 10% casamino acids; 10 ml of 20% glucose; 200 ml of 1% thiamine HCl; 200 ml of 5! Minimal Broth, Davis (MBD: 35 g dipotassium phosphate, 10 g monopotassium phosphate, 2.5 g sodium citrate, 0.5 g magnesium sulfate, and 5 g ammonium sulfate, per liter); 10 ml of Thy Pool (2 mg/ml of each of the following amino acids: L-Arg, L-His, L-Leu, L-Met, L-Pro, and L-Thr); and 1 ml of 0.1 M CaCl2, pH 7.0) supplemented with 100 mg/ml ampicillin.[26] For growth assays, 200 mL of the diluted cells were transferred into each well of a 96-well microtiter plate, and each well was supplemented with 2 ml of test compounds dissolved in dimethyl sulfoxide (DMSO). The concentration of the test compound in DMSO was adjusted such that the total concentration of DMSO in each well was the same and only the concentration of test compounds changed for purpose of producing dose-response curve fittings and calculating half maximal effective concentration (EC50) of each test compound. The 96-well microtiter plates were then incubated at 34°C, 150 rpm agitation, and 80 percent humidity for 16-24 hours.[26] Growth phenotypes of biosensor cells were then measured corresponding optical absorbance at a wavelength of 600 nanometers ($OD_{600}$) by utilizing Biotek Synergy 2 plate reader. All the bacterial assays were carried out in duplicate and were additionally repeated two times on two different days. The normalized growth signal is the difference

between the $OD_{600}$ value in the presence of a test compound and that in the presence of pure DMSO control for each strain.[4, 6, 26] The EC50 values was determined by fitting the growth data to equation (1), where X is the test compound concentration, Y is the observed growth signal ($OD_{600}$), and Bottom, Top, EC50, and Hillslope are the fitted parameters. The fitting was carried out with non-linear regression fitting by using the software package GraphPad Prism 5.01 (www.graphpad.com)[4, 6, 26].

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{\log(EC50 - X) \times HillSlope}} \qquad (1)$$

The statistical significances (P-value) between the $OD_{600}$ and EC50 were determined by using t-test function (two-tailed, two-sample equal variance) in jmp software (www.jmp.com). Relative pseudotransactivation (RPTA) values for each test compounds are calculated by using equation (2), where $EC^{E2}_{50}$ is the half maximal effective concentration of 17-β- estradiol, and $EC^{ligand}_{50}$ is the half maximal effective concentration of test compound. [4, 6]

$$EC_{50}^{E2} / EC_{50}^{ligand} \qquad (2)$$

## Results

### Detection of Estrogen with Current Biosensors

To test if phenotypic changes can be made by ERβ biosensors when EDCs are present, current biosensors were tested with E2, which is one of well-known EDCs. Positive control consists of E2 with DMSO and negative controls consist of DMSO and no ligand. For this control experiment, only biosensors of human (pMIT::ERβ*(h)), sole (pMIT::ERβ*(s)), and porcine (pMIT::ERβ*(p)) were employed. OD600 of each strains with its positive and negative controls were calculated by GraphPad Prism 5.01 and are shown in figure 4 below. All of the positive and the negative control OD600 result with all of available strains are reported in appendix I.

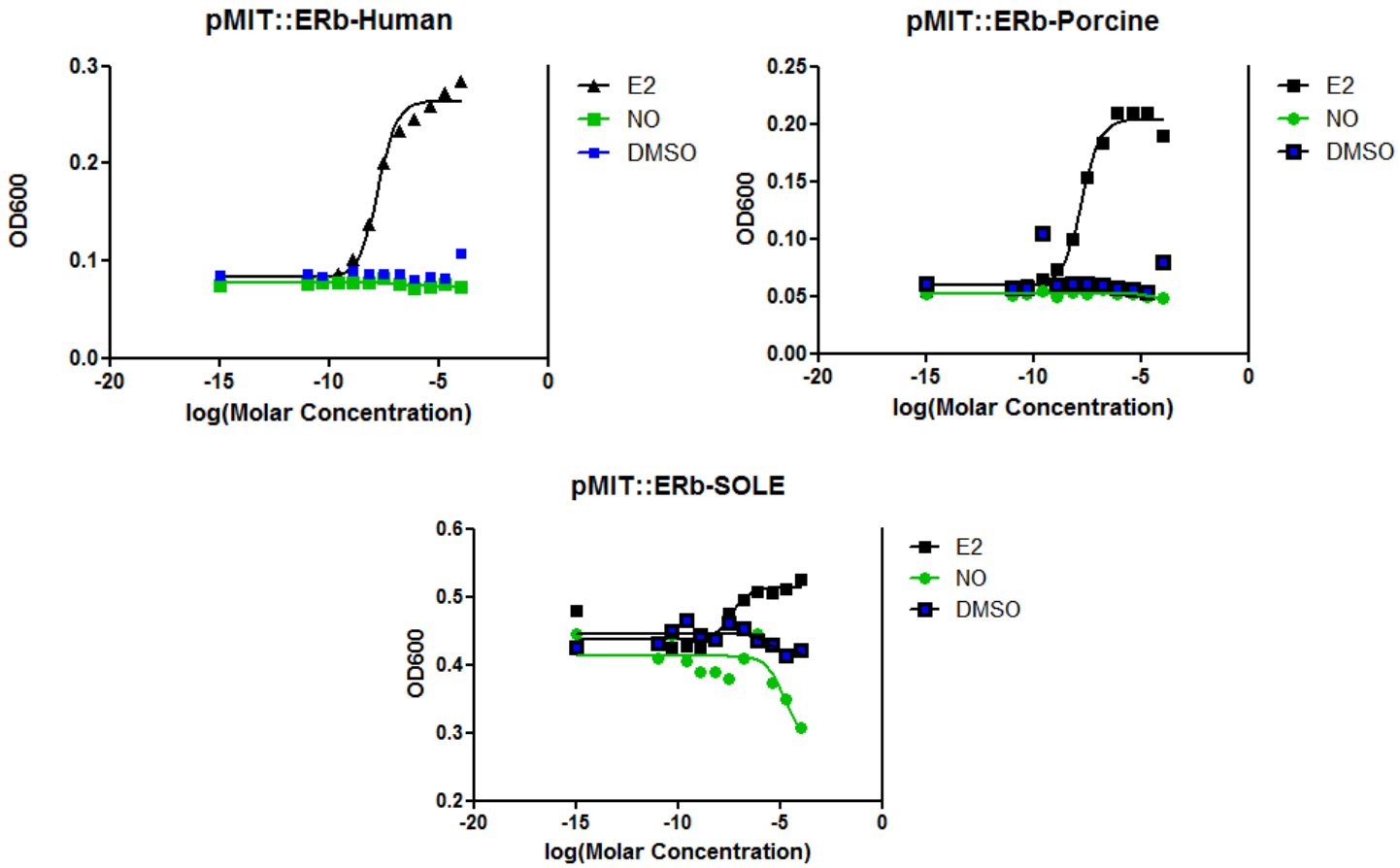



*Figure 4. Dose-response curves of pMIT::ERβ – Human, Porcine, and Sole. Curves were generated through non-linear regression fitting by GraphPad Prism 5.01 software. Notations are as follows: E2 = 17-β-estradiol, NO = no test ligand, DMSO = dimethyl sulfoxide*

When these biosensors are induced with E2, their growth phenotype changes were observed across these 3 different species. Thus, EC50 of E2 for human, sole, and porcine biosensors were able to be calculated. However, no significant growth phenotype changes were observed when biosensors were induced with DMSO and no test compound. As a result, EC50 could not be accurately calculated.

**Comparison of Current Biosensors**

EC50 and $OD_{600}$ measurements of *E.coli* biosensor of pMIT::ERβ*(h), pMIT::ERβ*(s), and pMIT::ERβ*(p), were compared with previously reported values for validation purposes. Table 1, 2, and 3 below shows the previously reported values (Gierach et al., 2011) and two other experimental values, respectively.

**Table 1. Prevously Reported EC50 and RPTA Values from Journal of Enviormental Toxicology (Gierach et al.) of Human, Porcine, and Sole Biosensor with E2, Daidzein and BPA in 2011.**

| ***Homo sapien*** **(Human) - Reported** | | | | |
|---|---|---|---|---|
| Ligand | EC50 (μM) | CI (μM) | R Square | RPTA(%) |
| **E2** | **0.015** | **0.00675 to 0.033** | **0.900** | **100** |
| **Daidzein** | **0.360** | **0.131 to 0.983** | **0.870** | **4.17** |
| **BPA** | **1.700** | **0.183 to 17.4** | **0.820** | **0.882** |
| | | | | |
| ***Sus scrofa*** **(Porcine) - Reported** | | | | |
| Ligand | EC50 (μM) | CI (μM) | R Square | RPTA(%) |
| **E2** | **0.016** | **0.013 to 0.0192** | **0.990** | **100** |
| **Daidzein** | **0.410** | **0.397 to 0.535** | **0.980** | **3.90** |
| **BPA** | **6.900** | **4.88 to 9.71** | **0.990** | **0.232** |
| | | | | |
| ***Solea solea*** **(Sole) - Reported** | | | | |
| Ligand | EC50 (μM) | CI (μM) | R Square | RPTA(%) |
| **E2** | **0.056** | **0.0209 to 0.153** | **0.860** | **100** |
| **Daidzein** | **6.000** | **3.25 to 11.4** | **0.910** | **0.933** |
| **BPA** | **1.000** | **0.107 to 9.85** | **0.860** | **5.60** |

**Table 2. First Experimental Values of EC50 and RPTA of Human, Porcine, and Sole Biosensor with E2, Daidzein and BPA.**

| *Homo sapien* (Human) - Experimental 1 | | | | |
|---|---|---|---|---|
| Ligand | EC50 (µM) | CI(µM) | R Square | RPTA(%) |
| E2 | 0.012 | 0.003 to 0.045 | 0.921 | 100 |
| Daidzein | 0.332 | 0.146 to 0.755 | 0.963 | 3.74 |
| BPA | 1.160 | 0.626 to 2.15 | 0.977 | 1.070 |

| *Sus scrofa* (Porcine) - Experimental 1 | | | | |
|---|---|---|---|---|
| Ligand | EC50 (µM) | CI(µM) | R Square | RPTA(%) |
| E2 | 0.020 | 0.0112 to 0.0337 | 0.984 | 100 |
| Daidzein | 0.571 | 0.424 to 0.769 | 0.995 | 3.42 |
| BPA | 7.103 | 4.75 to 10.6 | 0.990 | 0.275 |

| *Solea solea* (Sole) - Experimental 1 | | | | |
|---|---|---|---|---|
| Ligand | EC50 (µM) | CI(µM) | R Square | RPTA(%) |
| E2 | 0.047 | 0.00274 to 0.0810 | 0.698 | 100 |
| Daidzein | 9.865 | 0.840 to 11.6 | 0.717 | 0.478 |
| BPA | 1.144 | 0.816 to 5.60 | 0.772 | 4.12 |

**Table 3. Second Experimental Values of EC50 and RPTA of Human, Porcine, and Sole Biosensor with E2, Daidzein and BPA.**

| *Homo sapien* (Human) - Experimental 2 | | | | |
|---|---|---|---|---|
| Ligand | EC50 (μM) | CI | R Square | RPTA(%) |
| E2 | 0.018 | 0.0107 to 0.0287 | 0.987 | 100 |
| Daidzein | 0.357 | 0.235 to 0.528 | 0.991 | 4.90 |
| BPA | 2.108 | 1.34 to 3.32 | 0.987 | 0.831 |

| *Sus scrofa* (Porcine) - Experimental 2 | | | | |
|---|---|---|---|---|
| Ligand | EC50 (μM) | CI | R Square | RPTA(%) |
| E2 | 0.017 | 0.0113 to 0.0266 | 0.991 | 100 |
| Daidzein | 0.973 | 0.538 to 1.76 | 0.979 | 1.78 |
| BPA | 6.494 | 4.19 to 10.1 | 0.988 | 0.267 |

| *Solea solea* (Sole) - Experimental 2 | | | | |
|---|---|---|---|---|
| Ligand | EC50 (μM) | CI | R Square | RPTA(%) |
| E2 | 0.045 | 0.00709 to 0.279 | 0.847 | 100 |
| Daidzein | 6.261 | 5.33 to 7.36 | 0.998 | 0.711 |
| BPA | 1.365 | 0.949 to 1.96 | 0.992 | 3.26 |

From previously reported values, calculated EC50, 95% CI, and $R^2$ values are as follows: For (a) E2, Human EC50 = 0.015 μM (95% CI: 0.0067 μM to 0.033 μM; $R^2$: 0.900); Porcine EC50 = 0.016 μM (95% CI: 0.013 μM to 0.019 μM; $R^2$: 0.990); Sole EC50 = 0.056 μM (95% CI: 0.021 μM to 0.153 μM; $R^2$: 0.860 ); For (b) Daidzein, Human EC50 = 0.360 μM (95% CI: 0.130 μM to 0.980 μM; $R^2$: 0.870); Porcine EC50 =0.410 μM (95% CI: 0.390 μM to 0.535 μM; $R^2$: 0.980); Sole EC50 = 6.00μM (95% CI: 3.25 μM to 11.0 μM; $R^2$: 0.910); and for (c) BPA, Human EC50 = 1.70 μM (95% CI: 0.180 μM to 17.0 μM; $R^2$:0.820); Porcine EC50 = 6.90 μM (95% CI: 4.88 μM to 9.71 μM; $R^2$: 0.990 ); Sole EC50 = 1.00 μM (95% CI: 0.100 μM to 9.85 μM; $R^2$: 0.860).

For the reported first experimental values, EC50, 95% Confidence Interval, and $R^2$ values are as follows: For (a) E2, Human EC50 = 0.012 μM (95% CI: 0.003 μM to 0.045 μM; $R^2$: 0.921); Porcine EC50 = 0.020 μM (95% CI: 0.011 μM to 0.034 μM; $R^2$: 0.990); Sole EC50 = 0.047 μM (95% CI: 0.003 μM to 0.081 μM; $R^2$: 0.698); For (b) Daidzein, Human EC50 = 0.332 μM (95% CI: 0.146 μM to 0.755 μM; $R^2$: 0.963); Porcine EC50 =0.571 μM (95% CI: 0.424 μM to 0.769 μM; $R^2$: 0.980); Sole EC50 = 9.86μM (95% CI: 0.840 μM to 11.6 μM; $R^2$: 0.717); and for (c) BPA, Human EC50 = 1.16 μM (95% CI: 0.626 μM to 2.15 μM; $R^2$: 0.977); Porcine EC50 = 7.10 μM (95% CI:

4.75 µM to 10.6 µM; $R^2$: 0.990 ); Sole EC50 = 1.14 µM (95% CI: 0.816 µM to 5.60 µM; $R^2$: 0.772).

For the reported second experimental values, EC50, 95% Confidence Interval, and $R^2$ values are as follows: For (a) E2, Human EC50 = 0.018 µM (95% CI: 0.011 µM to 0.0287 µM; $R^2$: 0.987); Porcine EC50 = 0.017 µM (95% CI: 0.0113 µM to 0.0266 µM; $R^2$: 0.991); Sole EC50 = 0.045 µM (95% CI: 0.007 µM to 0.279 µM; $R^2$: 0.847); For (b) Daidzein, Human EC50 = 0.357 µM (95% CI: 0.235 µM to 0.528 µM; $R^2$: 0.991); Porcine EC50 =0.973 µM (95% CI: 0.538 µM to 1.76 µM; $R^2$: 0.979); Sole EC50 = 6.26 µM (95% CI: 5.33 µM to 7.36 µM; $R^2$: 0.998); and for (c) BPA, Human EC50 = 2.11 µM (95% CI: 1.34 µM to 3.32 µM; $R^2$: 0.987); Porcine EC50 = 6.49 µM (95% CI: 4.19 µM to 10.1 µM; $R^2$: 0.988 ); Sole EC50 = 1.37 µM (95% CI: 0.949 µM to 1.96 µM; $R^2$: 0.992).

Comparison between experimental values with the previously reported values were made and all of the values fall within the 95% confidence intervals of corresponding strains and its test compounds. For experimental values, RPTA precedence from the highest to lowest percentages are as follows: for Human and Porcine, E2 > Daidzein > BPA; for sole, E2 > BPA > Daidzein. This estrogenic potency precedence is the same with previously reported values.

**Detection of Phytoestrogens**

Phytoestrogens were used to induce the growth phenotype changes across *E.coli* biosensors of human, porcine, cow, rat, zebra fish, and sole. Dose-response curves are shown in figures 5 – 10 on the next page. Tables of calculated EC50 of phytoestrogens are shown in appendix II.

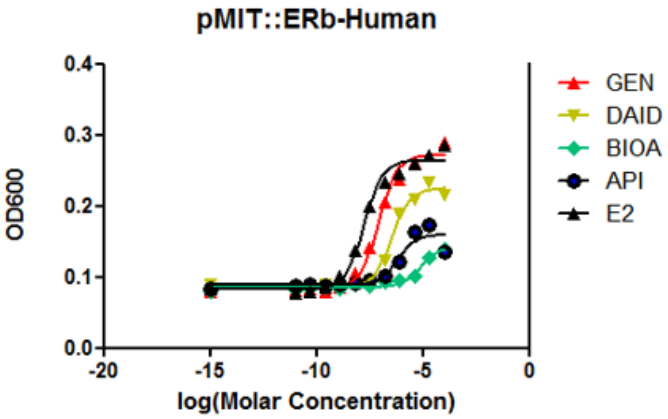



*Figure 5. A dose-response curve of human ERβ biosensor induced with various phytoestrogens*

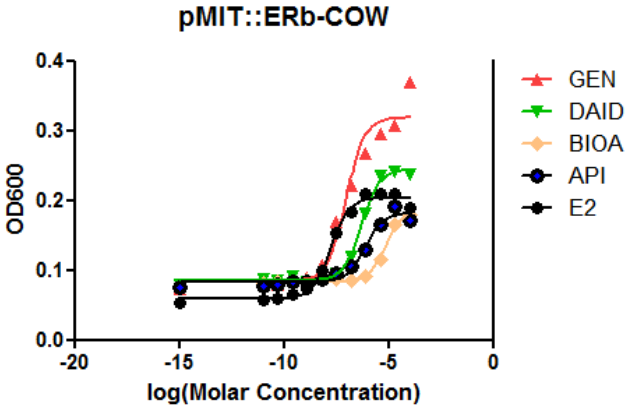



*Figure 6. A dose-response curve of cow ERβ biosensor induced with various phytoestrogens*

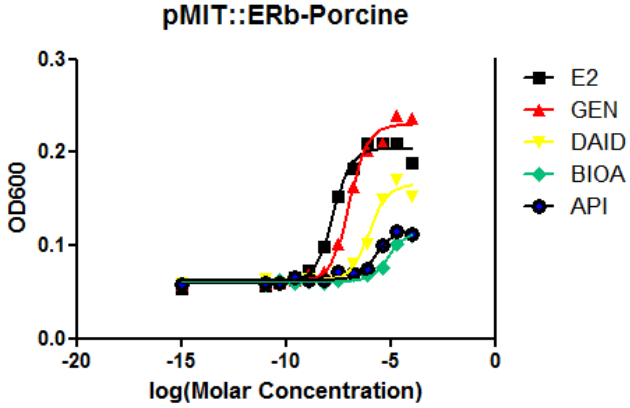



*Figure 7. A dose-response curve of porcine ERβ biosensor induced with various phytoestrogens*

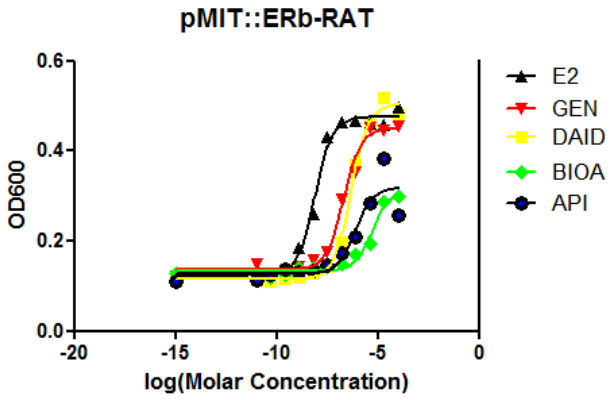



*Figure 8. A dose-response curve of rat ERβ biosensor induced with various phytoestrogens*

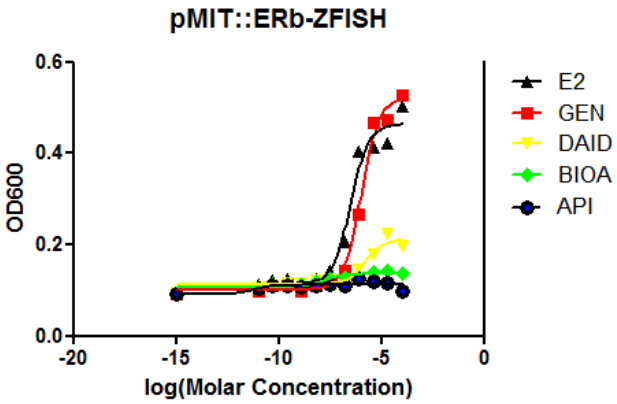



*Figure 9. A dose-response curve of zebra fish ERβ biosensor induced with various phytoestrogens*

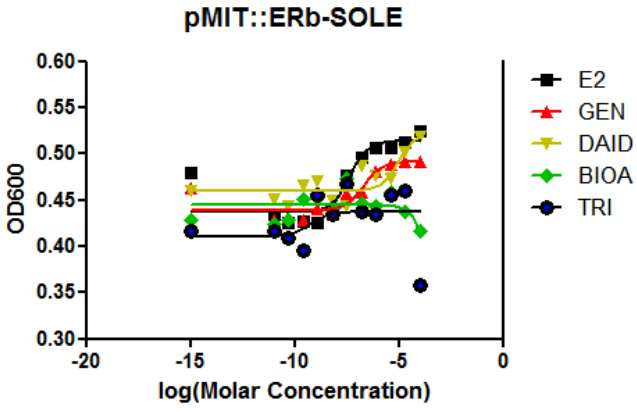



*Figure 10. A dose-response curve of sole ERβ biosensor induced with various phytoestrogens*

# Discussion

## Biosensor

In this study, an innovative approach for examining differences in agonistic ligand effects on the estrogen receptor β (ERβ) ligand binding domain derived from human, porcine, cow, rat, sole, and zebra fish were investigated. The key components of these methods are the engineered allosteric biosensor fusion proteins that are coupled with the thymidylate synthase (TS) reporter system. Previously, Dr. David W. Wood's group have demonstrated that these biosensors are capable of recognizing hormone-like compounds for various human NHR targets and can differentiate between agonistic and antagonistic compounds. [4, 6]

This biosensor system is very distinct from conventional transactivation assays, which require the presence of coregulators and detection systems for various reporter genes. [6] However, coregulators are not present in our system. Thus, ligand binding to the ERβ LBD produces a conformational change, which can be quantified by the TS reporter effects on phenotype. [4, 6] Furthermore, we can refer our system as a pseudotransactivation assay because the previous work with human NHR LBDs suggest that these conformational changes are related with those that lead to coactivation recruitment. [4, 6]

## Validation and Comparison of the Biosensor

Our results of biosensor proteins for human, porcine, and sole strains were compared with other results that were previously reported for validation purposes (Gierach et al., 2011; Hartman et al., 2009; Skretas et al., 2007; Skretas and Wood, 2005;). In addition, our results were also compared to studies that performed using a yeast-based human ERβ transcriptional assay indicate the following EC50 trend for our test compound: E2 > Daidzein > BPA (Escande et al., 2006). [28] Our RPTA trend is similar for human: E2 (100) > Daidzein (3.74) > BPA (1.07). Moreover, our results indicating that BPA is a weak human ERβ agonist are consistent with extensive previous work from other investigations (Kuiper et al., 1998; Shi et al., 2001; Kurosawa et al., 2002; Nendza and Wenzel, 2006; Dobbins et al., 2008; Chu et al., 2009). Most importantly, EC50 values of our bacterial biosensors assay were also compared to other previous studies with yeast assay for BPA and Daidzein: 1.7 µM (bacterial) versus 0.25 µM (yeast) and 0.36 µM (bacterial) versus 0.15 µM (yeast), respectively (Chu et al., 2009).[29]

Due to lack of available data, comparisons of our system with animal models are very difficult. However, a comparison was made to some closely related animal species. For example, several studies on other piscine species, such as carp (*Cyprinus carpio)* and rainbow trout *(oncorhynchus mykiss)* were used as a basis when comparing with

our sole biosensor results. These assays include direct binding assays on recombinant receptors that have been purified, along with transcriptional assays in yeast and human cells, and assays based on estrogen-induced secretion of vitellogenin in piscine hepatocytes. [32-34] The direct comparison of previously reported and our EC50 of sole biosensor is much different; however, consistent with the overall weakness of BPA as a ligand with the sole ERβ biosensor. For E2, previously reported EC50 varied between 50 and 150 nM [33], which is somewhat close with our EC50 variability of 2.7 to 81 for sole ERβ biosensor. In the same previously reported study, trout ERβ have shown the strongest affinity for E2 when compared with BPA and Daidzein, which were found to bind very weakly to the trout ERβ.[33] Note, only human and sole ERβ biosensor were able to be compared with other data.

**Overall Trend of Phytoestrogens in our ERβ Biosensors**

Isoflavones, such as genistein and daidzein, have been identified as angiogenesis inhibitors and likely to be found to inhibit the uncontrolled cell growth of cancer. This is most likely by inhibiting the activity of substances in the body that regulate cell division can cell growth factors. Furthermore, phytoestrogen such as genistein and other forms of isoflavones and flavones possess structure similarity to 17-β-estradiol (E2). Thus, these phytoestrogens such as genistein, daidzein, biochanin A, and apigenin may compete with E2 in binding to estrogen receptors. Out of four phytoestrogens that were tested, it was expected to observe the lowest EC50 with genistein because genistein shows much higher binding affinity toward estrogen receptor β in the previous study conducted with phytoestrogens (Kuiper et al., 1998).[30]

The estrogenicity precedence from highest to lowest is correlated with RPTA precedence from highest percentage to lowest percentage. Because each phytoestrogen in each bacterial assay has reported a distinct EC50 value, it is assumed that our bacterial biosensor for each species has correctly identified different phytoestrogens. The order of RPTA precedence of phytoestrogens for (a) human and (b)porcine biosensors are reported as follows: genistein > daidzein > apigenin > biochanin A; for (c) sole is reported as follows: genistein > daidzein; for (d) rat: genistein > apigenin > daidzein > biochanin A; for (e) cow: genistein > daidzein > apigenin > biochanin A; and for (f) zebra fish: daidzein > biochanin A > genistein. Calculated EC50 values for each different phytoestrogen in each ERβ biosensor were statistically determined to be significantly different. Thus, our experimental data suggests that genistein was the most potent phytoestrogen for human, porcine, sole, rat, and cow. However, EC50 of E2 is reported to be smaller than EC50 of genistein (RPTA percentage value for E2 is greater than that of genistein). This suggestion is consist with previous investigation by Kuiper et al. in 1998. Our results also suggests that biochanin A binds weakly to human, porcine, rat, and cow ERβ biosensor when compared to other three phytoestrogen that were being tested.

**Statistical Analysis of EC50 across Biosensor Species**

For evaluating the statistical significance between all of ERβ biosensor species, experimental EC50 were reported to jmp software and employed Fit Y by X function. A simple table is constructed for comparison of p-values with phytoestrogen EC50 values compared within each ERβ biosensor. This is shown in table 4 below. For all t-tests and tukey test that were conducted, alpha was set at 0.05.

**Table 4. Statistical analysis of EC50 of phytoestrogen across multiple species**

| ***HUMAN*** | **Genistein** | **Daidzein** | **Biochanin A** | **Apigenin** |
|---|---|---|---|---|
| **Genistein** | | 0.6377 | <0.0001 | 0.1564 |
| **Daidzein** | 0.6377 | | <0.0001 | 0.7058 |
| **Biochanin A** | <0.0001 | <0.0001 | | <0.0001 |
| **Apigenin** | 0.1564 | 0.7058 | <0.0001 | |

| ***PORCINE*** | **Genistein** | **Daidzein** | **Biochanin A** | **Apigenin** |
|---|---|---|---|---|
| **Genistein** | | 0.2845 | <0.0001 | 0.0036 |
| **Daidzein** | 0.2845 | | <0.0001 | 0.0939 |
| **Biochanin A** | <0.0001 | <0.0001 | | <0.0001 |
| **Apigenin** | 0.0036 | 0.0939 | <0.0001 | |

| ***SOLE*** | **Genistein** | **Daidzein** | **Biochanin A** | **Apigenin** |
|---|---|---|---|---|
| **Genistein** | | 0.0004 | N/A | N/A |
| **Daidzein** | 0.0004 | | N/A | N/A |
| **Biochanin A** | N/A | N/A | | N/A |
| **Apigenin** | N/A | N/A | N/A | |

| ***RAT*** | **Genistein** | **Daidzein** | **Biochanin A** | **Apigenin** |
|---|---|---|---|---|
| **Genistein** | | 0.0409 | <0.0001 | 0.1566 |
| **Daidzein** | 0.0409 | | <0.0001 | 0.4511 |
| **Biochanin A** | <0.0001 | <0.0001 | | <0.0001 |
| **Apigenin** | 0.1566 | 0.4511 | <0.0001 | |

| ***COW*** | **Genistein** | **Daidzein** | **Biochanin A** | **Apigenin** |
|---|---|---|---|---|
| **Genistein** | | <0.0001 | <0.0001 | <0.0001 |
| **Daidzein** | <0.0001 | | <0.0001 | 0.08031 |
| **Biochanin A** | <0.0001 | <0.0001 | | <0.0001 |
| **Apigenin** | <0.0001 | 0.0831 | <0.0001 | |

| ***ZFISH*** | **Genistein** | **Daidzein** | **Biochanin A** | **Apigenin** |
|---|---|---|---|---|
| **Genistein** | | 0.0014 | 0.004 | N/A |
| **Daidzein** | 0.0014 | | 0.7428 | N/A |
| **Biochanin A** | 0.004 | 0.7428 | | N/A |
| **Apigenin** | N/A | N/A | N/A | |

**Problems, Errors, Shortcomings, and Possible Future Projects**

Possible sources of error may have originated from utilizing materials that have been unsuccessfully sterilized; hindered cell growth during overnight and overday procedures; temperatures during cell growth may have not been consistent throughout the experiment; and incorrect distribution and concentration of ERβ biosensor cells and media.

Some of the EC50 of phytoestrogens were not able to be calculated due to the incorrect non-linear regression fitting. This is due to the human error of dispersing correct concentration of cells from a reservoir to a 96-microtitler well plate. This is seen in graphs where higher logarithmic values of molar concentration displays a lower OD600 reading when compared to OD600 readings of the lower logarithmic values of molar concentration of testing compounds.

The zebra fish ERβ biosensor were able to grow in the presence of phytoestrogens; however, only the wells in the plate with high concentration of phytoestrogen present were able to grow (log (molar concentration) of -7 or higher). Thus, this problem may have been avoided if observation were made after 16-24 hour interval.

The sole ERβ biosensor was much difficult to derive a definite conclusion, as OD600 values were unusually high. This is because we assume that sole LBD may be somewhat unfolded in the absence of any test compounds. As a result, unfolded LBD can destabilize the intein and allow higher background activity of the TS enzyme; thus, high OD600 readings may be observed. All of the OD600 readings with corresponding test compounds of all of strains performed in this study are reported in appendix I.

Possible future project may consist of finding more studies on binding assays with unknown estrogenic compounds, testing our bacterial assay with other endocrine disrupting compounds, and more.

## Acknowledgment

I gratefully acknowledge and thank Dr. David W. Wood, Department of Chemical and Biomolecular Engineering for providing immense amount of knowledge of bacterial biosensors, as well as the materials and funding for this project. I would also like to acknowledge Changhua Shi of Donghua University in Shanghai, China for his generosity of teaching anatomy and mechanism of bacterial biosensors.

## References


[1] Aranda, A. & Pascual, A. (2001). Nuclear hormone receptors and gene expression. Physiol. Rev. 81, 1269–1304.

[2] Bourguet, W., Germain, P. & Gronemeyer, H. (2000). Nuclear receptor ligand-binding domains: threedimensional structures, molecular interactions and pharmacological implications. Trends Pharmacol. Sci. 21, 381–388.

[3] Riggs, B. L. & Hartmann, L. C. (2003). Selective estrogen-receptor modulators – mechanisms of action and application to clinical practice. N. Engl. J. Med. 348, 618–629.

[4] Skretas, Georgios, and David W. Wood. "A bacterial biosensor of endocrine modulators." Journal of molecular biology 349.3 (2005): 464-474.

[5] Joyeux, A., Balaguer, P., Germain, P., Boussioux, A. M., Pons, M. & Nicolas, J. C. (1997). Engineered cell lines as a tool for monitoring biological activity of hormone analogs. Anal. Biochem. 249, 119–130

[6] Gierach, Izabela, et al. "Bacterial biosensors for evaluating potential impacts of estrogenic endocrine disrupting compounds in multiple species." *Environmental toxicology* 28.4 (2013): 179-189.

[7]Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N. & Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Health Perspect. 103, 113–122.

[8]Mattioni, T., Louvion, J. F. & Picard, D. (1994). Regulation of protein activities by fusion to steroid binding domains. Methods Cell Biol. 43, 335–352.

[9] Picard, D. (2000). Posttranslational regulation of proteins by fusions to steroid-binding domains. Methods Enzymol. 327, 385–401.

[10] Tucker, C. L. & Fields, S. (2001). A yeast sensor of ligand binding. Nature Biotechnol. 19, 1042–1046.

[11] Carreras CW, and Santi DV (1995). "The Catalytic Mechanism and Structure of Thymidylate Synthase". Annual Review of Biochemistry 64 (1): 721–762. doi:10.1146/annurev.bi.64.070195.003445. PMID 7574499.

[12] Derbyshire, V., Wood, D. W., Wu, W., Dansereau, J. T., Dalgaard, J. Z. & Belfort, M. (1997). Genetic definition of a protein-splicing domain: functional mini-inteins support structure predictions and a model for intein evolution. Proc. Natl Acad. Sci. USA, 94, 11466–11471.

[13] Wood, D. W., Wu, W., Belfort, G., Derbyshire, V. & Belfort, M. (1999). A genetic system yields selfcleaving inteins for bioseparations. Nature Biotechnol. 17, 889–892.

[14] Srinivasan, G. (1992). Overexpression of receptors of the steroid/thyroid family. Mol. Endocrinol. 6, 857–860.

[15] Wooge, C. H., Nilsson, G. M., Heierson, A., McDonnell, D. P. & Katzenellenbogen, B. S. (1992). Structural requirements for high affinity ligand binding by estrogen receptors: a comparative analysis of truncated and full length estrogen receptors expressed in bacteria, yeast, and mammalian cells. Mol. Endocrinol. 6, 861–869.

[16] Power, R. F., Conneely, O. M., McDonnell, D. P., Clark, J. H., Butt, T. R., Schrader, W. T. & O0Malley, B. W. (1990). High level expression of a truncated chicken progesterone receptor in Escherichia coli. J. Biol. Chem. 265, 1419–1424.

[17] Wittliff, J. L., Wenz, L. L., Dong, J., Nawaz, Z. & Butt, T. R. (1990). Expression and characterization of an active human estrogen receptor as a ubiquitin fusion protein from Escherichia coli. J. Biol. Chem. 265, 2016–22022.

[18]Power, R. F., Conneely, O. M., McDonnell, D. P., Clark, J. H., Butt, T. R., Schrader, W. T. & O0Malley, B. W. (1990). High level expression of a truncated chicken progesterone receptor in Escherichia coli. J. Biol. Chem. 265, 1419–1424.

[19]Paulus, H. (2003). Inteins as targets for potential antimycobacterial drugs. Front. Biosci. 8, s1147–s1165.

[20] Gangopadhyay, J. P., Jiang, S. Q., van Berkel, P. & Paulus, H. (2003). In vitro splicing of erythropoietin by the Mycobacterium tuberculosis RecA intein without substituting amino acids at the splice junctions. Biochim. Biophys. Acta, 1619, 193–200.

[21]Wu, W., Wood, D. W., Belfort, G., Derbyshire, V. & Belfort, M. (2002). Intein-mediated purification of cytotoxic endonuclease I-TevI by insertional inactivation and pH-controllable splicing. Nucl. Acids Res. 30, 4864–4871.

[22]Fitzsimons Hall, M., Noren, C. J., Perler, F. B. & Schildkraut, I. (2002). Creation of an artificial bifunctional intein by grafting a homing endonuclease into a mini-intein. J. Mol. Biol. 323, 173–179.

[23] Buskirk, A. R., Ong, Y. C., Gartner, Z. J. & Liu, D. R. (2004). Directed evolution of ligand dependence: small-molecule-activated protein splicing. Proc. Natl Acad. Sci. USA, 101, 10505–10510.

[24] Skretas, G. & Wood, D. W. (2005). Regulation of protein activity with small-molecule-controlled inteins. Protein Sci. 14, 523–532.

[25] Kapust, R. B. & Waugh, D. S. (1999). Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. Protein Sci. 8, 1668–1674.

[26] Changhua Shi, Qing Meng, David W Wood Analysis of the roles of mutations in thyroid hormone receptor-β by a bacterial biosensor system Mol Endocrinol 52 (1) 55-66, doi: 10.1530/JME-13-0108 first published on 30 October 2013

[27] Hartman I, Gillies AR, Arora S, Andaya C, Royapet N, Welsh WJ, Wood DW, Zauhar RJ. 2009. Application of screening methods, shape signatures and engineered biosensors in early drug discovery process. Pharm Res 26:2247–2258.

[28] Escande A, Pillon A, Servant N, Cravedi JP, Larrea F, Muhn P, Nicolas JC, Cavailles V, Balaguer P. 2006. Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. Biochem Pharmacol 71:1459–1469.

[29] Chu WL, Shiizaki K, Kawanishi M, Kondo M, Yagi T. 2009. Validation of a new yeast-based reporter assay consisting of human estrogen receptors alpha/beta and coactivator SRC-1: Application for detection of estrogenic activity in environmental samples. Environmental Toxicology 24:513–521.

[30] Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252–4263.

[31] Shi LM, Fang H, Tong W, Wu J, Perkins R, Blair RM, Branham WS, Dial SL, Moland CL, Sheehan DM. 2001. QSAR models using a large diverse set of estrogens. J Chem Inf Comput Sci 41:186–195.

[32] Matthews J, Celius T, Halgren R, Zacharewski T. 2000. Differential estrogen receptor binding of estrogenic substances: A species comparison. J Steroid Biochem Mol Biol 74:223–234.

[33] Smeets JM, Rankouhi TR, Nichols KM, Komen H, Kaminski NE, Giesy JP, van den Berg M. 1999. In vitro vitellogenin production by carp (Cyprinus carpio) hepatocytes as a screening method for determining (anti)estrogenic activity of xenobiotics. Toxicol Appl Pharmacol 157:68–76.

[34] Petit F, Valotaire Y, Pakdel F. 1995. Differential functional activities of rainbow trout and human estrogen receptors expressed in the yeast Saccharomyces cerevisiae. Eur J Biochem 233:584–592.

# Appendix I

## Reported OD600 values

| HUMAN | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.073 | 0.076 | 0.073 | 0.072 | 0.076 | 0.082 | 0.077 | 0.077 | 0.078 | 0.078 | 0.076 | 0.075 |
| **E2** | 0.285 | 0.272 | 0.259 | 0.245 | 0.234 | 0.2 | 0.137 | 0.101 | 0.086 | 0.08 | 0.078 | 0.083 |
| **GEN** | 0.29 | 0.272 | 0.261 | 0.238 | 0.205 | 0.141 | 0.106 | 0.086 | 0.08 | 0.083 | 0.082 | 0.08 |
| **DAID** | 0.215 | 0.233 | 0.209 | 0.19 | 0.123 | 0.094 | 0.087 | 0.087 | 0.089 | 0.081 | 0.085 | 0.089 |
| **BIOA** | 0.139 | 0.127 | 0.102 | 0.096 | 0.093 | 0.088 | 0.089 | 0.084 | 0.086 | 0.083 | 0.081 | 0.08 |
| **TRI** | 0.136 | 0.174 | 0.163 | 0.121 | 0.102 | 0.095 | 0.091 | 0.09 | 0.088 | 0.089 | 0.088 | 0.084 |
| **DMSO** | 0.108 | 0.082 | 0.083 | 0.081 | 0.086 | 0.087 | 0.087 | 0.09 | 0.19 | 0.083 | 0.087 | 0.085 |
| **GEN** | 0.274 | 0.291 | 0.267 | 0.285 | 0.21 | 0.147 | 0.097 | 0.09 | 0.081 | 0.084 | 0.081 | 0.079 |

| HUMAN | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.079 | 0.071 | 0.073 | 0.073 | 0.08 | 0.083 | 0.084 | 0.083 | 0.086 | 0.083 | 0.084 | 0.074 |
| **E2** | 0.461 | 0.464 | 0.404 | 0.394 | 0.382 | 0.384 | 0.403 | 0.195 | 0.117 | 0.095 | 0.091 | 0.077 |
| **GEN** | 0.438 | 0.422 | 0.45 | 0.388 | 0.389 | 0.434 | 0.202 | 0.125 | 0.099 | 0.093 | 0.09 | 0.082 |
| **DAID** | 0.491 | 0.38 | 0.406 | 0.445 | 0.285 | 0.133 | 0.102 | 0.097 | 0.095 | 0.093 | 0.091 | 0.087 |
| **BIOA** | 0.317 | 0.303 | 0.147 | 0.111 | 0.102 | 0.097 | 0.095 | 0.095 | 0.098 | 0.09 | 0.087 | 0.079 |
| **TRI** | 0.33 | 0.483 | 0.47 | 0.321 | 0.157 | 0.109 | 0.101 | 0.097 | 0.102 | 0.096 | 0.086 | 0.08 |
| **DMSO** | 0.21 | 0.078 | 0.085 | 0.097 | 0.09 | 0.093 | 0.096 | 0.094 | 0.389 | 0.086 | 0.084 | 0.075 |
| **GEN** | 0.507 | 0.445 | 0.404 | 0.456 | 0.459 | 0.444 | 0.181 | 0.112 | 0.085 | 0.082 | 0.076 | 0.075 |

| HUMAN | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.073 | 0.076 | 0.076 | 0.078 | 0.09 | 0.091 | 0.089 | 0.089 | 0.092 | 0.09 | 0.092 | 0.077 |
| **E2** | 0.5 | 0.51 | 0.439 | 0.425 | 0.424 | 0.425 | 0.463 | 0.242 | 0.135 | 0.103 | 0.097 | 0.08 |
| **GEN** | 0.471 | 0.461 | 0.482 | 0.429 | 0.421 | 0.469 | 0.258 | 0.143 | 0.108 | 0.098 | 0.093 | 0.082 |
| **DAID** | 0.522 | 0.423 | 0.445 | 0.481 | 0.367 | 0.157 | 0.111 | 0.105 | 0.1 | 0.098 | 0.094 | 0.079 |
| **BIOA** | 0.378 | 0.387 | 0.176 | 0.121 | 0.108 | 0.104 | 0.101 | 0.101 | 0.108 | 0.096 | 0.092 | 0.091 |
| **TRI** | 0.389 | 0.512 | 0.499 | 0.402 | 0.189 | 0.119 | 0.105 | 0.102 | 0.113 | 0.097 | 0.098 | 0.087 |
| **DMSO** | 0.253 | 0.077 | 0.086 | 0.098 | 0.093 | 0.095 | 0.101 | 0.1 | 0.428 | 0.09 | 0.086 | 0.076 |
| **GEN** | 0.552 | 0.477 | 0.437 | 0.492 | 0.496 | 0.5 | 0.224 | 0.126 | 0.09 | 0.087 | 0.077 | 0.076 |

| COW | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.069 | 0.07 | 0.071 | 0.074 | 0.069 | 0.07 | 0.068 | 0.068 | 0.073 | 0.071 | 0.072 | 0.067 |
| **E2** | 0.339 | 0.28 | 0.224 | 0.117 | 0.089 | 0.083 | 0.072 | 0.074 | 0.074 | 0.073 | 0.072 | 0.07 |
| **GEN** | 0.37 | 0.307 | 0.295 | 0.268 | 0.222 | 0.169 | 0.108 | 0.089 | 0.085 | 0.079 | 0.08 | 0.074 |
| **DAID** | 0.237 | 0.241 | 0.236 | 0.181 | 0.119 | 0.096 | 0.086 | 0.083 | 0.092 | 0.086 | 0.087 | 0.08 |
| **BIOA** | 0.179 | 0.166 | 0.115 | 0.091 | 0.086 | 0.087 | 0.09 | 0.085 | 0.083 | 0.084 | 0.082 | 0.075 |
| **TRI** | 0.172 | 0.191 | 0.165 | 0.129 | 0.106 | 0.096 | 0.087 | 0.083 | 0.084 | 0.08 | 0.077 | 0.076 |
| **DMSO** | 0.116 | 0.077 | 0.079 | 0.079 | 0.086 | 0.086 | 0.084 | 0.084 | 0.193 | 0.084 | 0.08 | 0.082 |
| **BPA** | 0.149 | 0.115 | 0.083 | 0.077 | 0.08 | 0.078 | 0.091 | 0.077 | 0.081 | 0.074 | 0.075 | 0.072 |

| COW | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.086 | 0.068 | 0.072 | 0.072 | 0.075 | 0.073 | 0.068 | 0.069 | 0.073 | 0.073 | 0.073 | 0.067 |
| **E2** | 0.503 | 0.456 | 0.44 | 0.388 | 0.125 | 0.091 | 0.073 | 0.073 | 0.072 | 0.075 | 0.072 | 0.067 |
| **GEN** | 0.478 | 0.419 | 0.436 | 0.426 | 0.419 | 0.433 | 0.249 | 0.134 | 0.101 | 0.09 | 0.089 | 0.075 |
| **DAID** | 0.452 | 0.402 | 0.432 | 0.429 | 0.381 | 0.158 | 0.108 | 0.101 | 0.107 | 0.094 | 0.093 | 0.083 |
| **BIOA** | 0.495 | 0.414 | 0.336 | 0.148 | 0.114 | 0.105 | 0.101 | 0.097 | 0.091 | 0.095 | 0.09 | 0.073 |
| **TRI** | 0.445 | 0.45 | 0.484 | 0.426 | 0.217 | 0.129 | 0.107 | 0.096 | 0.101 | 0.085 | 0.083 | 0.075 |
| **DMSO** | 0.327 | 0.077 | 0.08 | 0.084 | 0.093 | 0.097 | 0.092 | 0.092 | 0.436 | 0.09 | 0.078 | 0.081 |
| **BPA** | 0.443 | 0.36 | 0.106 | 0.081 | 0.077 | 0.077 | 0.09 | 0.076 | 0.081 | 0.072 | 0.07 | 0.069 |

| COW | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.088 | 0.056 | 0.074 | 0.074 | 0.077 | 0.075 | 0.07 | 0.073 | 0.075 | 0.073 | 0.073 | 0.067 |
| **E2** | 0.523 | 0.519 | 0.475 | 0.438 | 0.141 | 0.097 | 0.075 | 0.076 | 0.075 | 0.077 | 0.073 | 0.067 |
| **GEN** | 0.514 | 0.452 | 0.472 | 0.471 | 0.462 | 0.469 | 0.3 | 0.15 | 0.11 | 0.094 | 0.091 | 0.076 |
| **DAID** | 0.493 | 0.437 | 0.466 | 0.47 | 0.444 | 0.181 | 0.121 | 0.113 | 0.113 | 0.099 | 0.099 | 0.083 |
| **BIOA** | 0.522 | 0.437 | 0.403 | 0.168 | 0.131 | 0.111 | 0.106 | 0.102 | 0.093 | 0.097 | 0.093 | 0.079 |
| **TRI** | 0.477 | 0.479 | 0.525 | 0.467 | 0.255 | 0.142 | 0.117 | 0.104 | 0.108 | 0.089 | 0.091 | 0.075 |
| **DMSO** | 0.38 | 0.08 | 0.085 | 0.094 | 0.097 | 0.103 | 0.098 | 0.097 | 0.492 | 0.092 | 0.082 | 0.083 |
| **BPA** | 0.449 | 0.185 | 0.117 | 0.082 | 0.077 | 0.071 | 0.115 | 0.045 | 0.047 | 0.043 | 0.042 | 0.07 |

| PORCINE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.048 | 0.05 | 0.052 | 0.052 | 0.056 | 0.052 | 0.053 | 0.05 | 0.055 | 0.052 | 0.051 | 0.052 |
| **E2** | 0.189 | 0.21 | 0.21 | 0.209 | 0.183 | 0.153 | 0.099 | 0.073 | 0.065 | 0.06 | 0.057 | 0.054 |
| **GEN** | 0.237 | 0.24 | 0.209 | 0.202 | 0.163 | 0.102 | 0.072 | 0.068 | 0.063 | 0.06 | 0.059 | 0.058 |
| **DAID** | 0.152 | 0.171 | 0.15 | 0.102 | 0.08 | 0.072 | 0.063 | 0.064 | 0.06 | 0.06 | 0.064 | 0.06 |
| **BIOA** | 0.11 | 0.102 | 0.076 | 0.068 | 0.068 | 0.062 | 0.06 | 0.061 | 0.06 | 0.062 | 0.058 | 0.058 |
| **TRI** | 0.112 | 0.115 | 0.1 | 0.074 | 0.07 | 0.072 | 0.063 | 0.062 | 0.066 | 0.059 | 0.06 | 0.058 |
| **DMSO** | 0.08 | 0.053 | 0.056 | 0.057 | 0.06 | 0.061 | 0.061 | 0.059 | 0.104 | 0.057 | 0.057 | 0.061 |
| **E2** | 0.178 | 0.149 | 0.115 | 0.089 | 0.067 | 0.063 | 0.06 | 0.06 | 0.057 | 0.055 | 0.056 | 0.056 |

| PORCINE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.047 | 0.048 | 0.051 | 0.053 | 0.053 | 0.052 | 0.053 | 0.051 | 0.053 | 0.053 | 0.052 | 0.051 |
| **E2** | 0.491 | 0.451 | 0.455 | 0.448 | 0.452 | 0.458 | 0.322 | 0.1 | 0.067 | 0.063 | 0.058 | 0.053 |
| **GEN** | 0.478 | 0.457 | 0.51 | 0.488 | 0.462 | 0.38 | 0.09 | 0.069 | 0.063 | 0.061 | 0.06 | 0.055 |
| **DAID** | 0.456 | 0.455 | 0.432 | 0.366 | 0.122 | 0.078 | 0.065 | 0.065 | 0.059 | 0.06 | 0.064 | 0.055 |
| **BIOA** | 0.285 | 0.342 | 0.113 | 0.07 | 0.068 | 0.066 | 0.063 | 0.062 | 0.061 | 0.061 | 0.059 | 0.056 |
| **TRI** | 0.288 | 0.443 | 0.343 | 0.129 | 0.078 | 0.08 | 0.063 | 0.062 | 0.064 | 0.058 | 0.058 | 0.057 |
| **DMSO** | 0.114 | 0.055 | 0.056 | 0.057 | 0.057 | 0.06 | 0.06 | 0.062 | 0.32 | 0.059 | 0.058 | 0.057 |
| **DAID** | 0.468 | 0.432 | 0.48 | 0.24 | 0.078 | 0.065 | 0.06 | 0.059 | 0.055 | 0.054 | 0.053 | 0.051 |

| PORCINE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.047 | 0.052 | 0.051 | 0.053 | 0.054 | 0.053 | 0.055 | 0.05 | 0.052 | 0.053 | 0.051 | 0.051 |
| **E2** | 0.562 | 0.498 | 0.503 | 0.497 | 0.494 | 0.484 | 0.417 | 0.115 | 0.068 | 0.063 | 0.058 | 0.054 |
| **GEN** | 0.516 | 0.501 | 0.56 | 0.523 | 0.496 | 0.482 | 0.099 | 0.071 | 0.064 | 0.061 | 0.059 | 0.057 |
| **DAID** | 0.474 | 0.497 | 0.475 | 0.457 | 0.146 | 0.081 | 0.066 | 0.067 | 0.059 | 0.058 | 0.064 | 0.057 |
| **BIOA** | 0.356 | 0.431 | 0.132 | 0.071 | 0.069 | 0.067 | 0.062 | 0.063 | 0.063 | 0.06 | 0.059 | 0.057 |
| **TRI** | 0.374 | 0.472 | 0.438 | 0.158 | 0.082 | 0.084 | 0.065 | 0.062 | 0.065 | 0.059 | 0.059 | 0.059 |
| **DMSO** | 0.13 | 0.057 | 0.056 | 0.056 | 0.057 | 0.059 | 0.059 | 0.061 | 0.411 | 0.057 | 0.057 | 0.056 |
| **E2** | 0.514 | 0.484 | 0.491 | 0.305 | 0.085 | 0.067 | 0.06 | 0.06 | 0.055 | 0.053 | 0.054 | 0.051 |

| RAT | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.081 | 0.084 | 0.085 | 0.091 | 0.108 | 0.097 | 0.094 | 0.096 | 0.092 | 0.089 | 0.086 | 0.087 |
| **E2** | 0.197 | 0.223 | 0.186 | 0.126 | 0.102 | 0.095 | 0.092 | 0.088 | 0.087 | 0.088 | 0.088 | 0.088 |
| **GEN** | 0.228 | 0.222 | 0.203 | 0.193 | 0.169 | 0.137 | 0.115 | 0.105 | 0.093 | 0.093 | 0.091 | 0.094 |
| **DAID** | 0.159 | 0.163 | 0.146 | 0.123 | 0.121 | 0.103 | 0.1 | 0.099 | 0.097 | 0.095 | 0.1 | 0.095 |
| **BIOA** | 0.125 | 0.116 | 0.098 | 0.108 | 0.093 | 0.097 | 0.096 | 0.098 | 0.093 | 0.091 | 0.087 | 0.098 |
| **TRI** | 0.115 | 0.118 | 0.116 | 0.106 | 0.102 | 0.1 | 0.098 | 0.096 | 0.096 | 0.094 | 0.095 | 0.093 |
| **DMSO** | 0.101 | 0.087 | 0.09 | 0.093 | 0.104 | 0.096 | 0.094 | 0.095 | 0.126 | 0.089 | 0.089 | 0.09 |
| **BPA** | 0.113 | 0.103 | 0.089 | 0.087 | 0.09 | 0.09 | 0.091 | 0.09 | 0.09 | 0.088 | 0.091 | 0.09 |

| RAT | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.157 | 0.123 | 0.119 | 0.138 | 0.186 | 0.153 | 0.143 | 0.152 | 0.135 | 0.124 | 0.12 | 0.112 |
| **E2** | 0.482 | 0.519 | 0.457 | 0.371 | 0.196 | 0.142 | 0.129 | 0.12 | 0.117 | 0.109 | 0.112 | 0.114 |
| **GEN** | 0.496 | 0.458 | 0.46 | 0.468 | 0.463 | 0.431 | 0.26 | 0.184 | 0.137 | 0.128 | 0.122 | 0.118 |
| **DAID** | 0.455 | 0.446 | 0.452 | 0.353 | 0.293 | 0.175 | 0.157 | 0.144 | 0.133 | 0.128 | 0.148 | 0.121 |
| **BIOA** | 0.3 | 0.286 | 0.194 | 0.169 | 0.15 | 0.144 | 0.14 | 0.141 | 0.124 | 0.119 | 0.117 | 0.129 |
| **TRI** | 0.256 | 0.382 | 0.284 | 0.208 | 0.173 | 0.147 | 0.134 | 0.132 | 0.138 | 0.126 | 0.112 | 0.109 |
| **DMSO** | 0.205 | 0.112 | 0.115 | 0.127 | 0.157 | 0.133 | 0.127 | 0.128 | 0.329 | 0.112 | 0.106 | 0.101 |
| **BPA** | 0.346 | 0.214 | 0.116 | 0.103 | 0.105 | 0.104 | 0.109 | 0.108 | 0.104 | 0.103 | 0.096 | 0.094 |

| SOLE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.307 | 0.349 | 0.374 | 0.445 | 0.41 | 0.38 | 0.389 | 0.389 | 0.406 | 0.444 | 0.41 | 0.445 |
| **E2** | 0.525 | 0.512 | 0.506 | 0.507 | 0.496 | 0.476 | 0.44 | 0.426 | 0.427 | 0.425 | 0.434 | 0.479 |
| **GEN** | 0.491 | 0.492 | 0.489 | 0.481 | 0.458 | 0.457 | 0.434 | 0.441 | 0.429 | 0.432 | 0.428 | 0.463 |
| **DAID** | 0.434 | 0.452 | 0.44 | 0.419 | 0.43 | 0.416 | 0.439 | 0.436 | 0.427 | 0.437 | 0.437 | 0.423 |
| **BIOA** | 0.417 | 0.438 | 0.458 | 0.443 | 0.447 | 0.474 | 0.441 | 0.452 | 0.451 | 0.428 | 0.424 | 0.429 |
| **TRI** | 0.358 | 0.46 | 0.456 | 0.434 | 0.438 | 0.467 | 0.435 | 0.455 | 0.395 | 0.409 | 0.417 | 0.417 |
| **DMSO** | 0.421 | 0.414 | 0.43 | 0.435 | 0.453 | 0.461 | 0.437 | 0.444 | 0.466 | 0.45 | 0.431 | 0.425 |
| **E2** | 0.507 | 0.51 | 0.481 | 0.488 | 0.479 | 0.464 | 0.422 | 0.44 | 0.433 | 0.407 | 0.452 | 0.428 |

| SOLE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.504 | 0.495 | 0.499 | 0.519 | 0.515 | 0.498 | 0.504 | 0.447 | 0.528 | 0.535 | 0.534 | 0.54 |
| **E2** | 0.58 | 0.578 | 0.525 | 0.52 | 0.519 | 0.505 | 0.485 | 0.48 | 0.474 | 0.481 | 0.521 | 0.575 |
| **GEN** | 0.587 | 0.535 | 0.526 | 0.499 | 0.491 | 0.49 | 0.483 | 0.477 | 0.48 | 0.481 | 0.493 | 0.524 |
| **DAID** | 0.529 | 0.48 | 0.472 | 0.473 | 0.502 | 0.471 | 0.483 | 0.499 | 0.476 | 0.476 | 0.484 | 0.49 |
| **BIOA** | 0.526 | 0.479 | 0.481 | 0.478 | 0.485 | 0.52 | 0.475 | 0.484 | 0.484 | 0.484 | 0.544 | 0.539 |
| **TRI** | 0.437 | 0.505 | 0.541 | 0.488 | 0.488 | 0.523 | 0.478 | 0.487 | 0.476 | 0.482 | 0.521 | 0.513 |
| **DMSO** | 0.534 | 0.474 | 0.475 | 0.49 | 0.539 | 0.487 | 0.486 | 0.482 | 0.508 | 0.493 | 0.496 | 0.494 |
| **DAID** | 0.578 | 0.583 | 0.535 | 0.534 | 0.524 | 0.511 | 0.489 | 0.486 | 0.529 | 0.499 | 0.509 | 0.49 |

| SOLE | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.522 | 0.514 | 0.512 | 0.528 | 0.523 | 0.517 | 0.522 | 0.472 | 0.54 | 0.542 | 0.548 | 0.56 |
| **E2** | 0.608 | 0.597 | 0.547 | 0.539 | 0.542 | 0.538 | 0.508 | 0.503 | 0.498 | 0.504 | 0.534 | 0.602 |
| **GEN** | 0.603 | 0.568 | 0.548 | 0.517 | 0.516 | 0.517 | 0.509 | 0.502 | 0.508 | 0.508 | 0.525 | 0.547 |
| **DAID** | 0.551 | 0.511 | 0.493 | 0.496 | 0.527 | 0.497 | 0.514 | 0.529 | 0.5 | 0.51 | 0.522 | 0.516 |
| **BIOA** | 0.539 | 0.509 | 0.511 | 0.507 | 0.507 | 0.546 | 0.504 | 0.508 | 0.51 | 0.521 | 0.569 | 0.558 |
| **TRI** | 0.454 | 0.53 | 0.569 | 0.514 | 0.512 | 0.548 | 0.506 | 0.514 | 0.503 | 0.511 | 0.535 | 0.526 |
| **DMSO** | 0.552 | 0.496 | 0.493 | 0.516 | 0.561 | 0.517 | 0.513 | 0.509 | 0.541 | 0.522 | 0.529 | 0.516 |
| **DAID** | 0.602 | 0.609 | 0.56 | 0.561 | 0.552 | 0.538 | 0.516 | 0.513 | 0.552 | 0.513 | 0.531 | 0.511 |

| ZFISH | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.085 | 0.086 | 0.095 | 0.091 | 0.096 | 0.094 | 0.086 | 0.084 | 0.095 | 0.083 | 0.08 | 0.079 |
| **E2** | 0.394 | 0.31 | 0.22 | 0.132 | 0.103 | 0.097 | 0.089 | 0.091 | 0.091 | 0.088 | 0.081 | 0.08 |
| **GEN** | 0.277 | 0.25 | 0.177 | 0.131 | 0.106 | 0.098 | 0.097 | 0.089 | 0.1 | 0.095 | 0.093 | 0.088 |
| **DAID** | 0.116 | 0.104 | 0.109 | 0.102 | 0.095 | 0.094 | 0.091 | 0.088 | 0.095 | 0.091 | 0.093 | 0.087 |
| **BIOA** | 0.11 | 0.102 | 0.107 | 0.095 | 0.092 | 0.092 | 0.095 | 0.086 | 0.092 | 0.091 | 0.089 | 0.088 |
| **TRI** | 0.092 | 0.092 | 0.097 | 0.103 | 0.097 | 0.099 | 0.091 | 0.09 | 0.091 | 0.09 | 0.089 | 0.085 |
| **DMSO** | 0.099 | 0.093 | 0.102 | 0.098 | 0.095 | 0.096 | 0.092 | 0.092 | 0.111 | 0.091 | 0.09 | 0.086 |
| **BPA** | 0.1 | 0.101 | 0.103 | 0.092 | 0.096 | 0.089 | 0.088 | 0.087 | 0.092 | 0.089 | 0.087 | 0.091 |

| ZFISH | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NO** | 0.105 | 0.102 | 0.122 | 0.122 | 0.13 | 0.125 | 0.112 | 0.104 | 0.128 | 0.113 | 0.105 | 0.091 |
| **E2** | 0.527 | 0.473 | 0.467 | 0.267 | 0.143 | 0.118 | 0.109 | 0.098 | 0.114 | 0.109 | 0.098 | 0.091 |
| **GEN** | 0.503 | 0.422 | 0.413 | 0.403 | 0.205 | 0.142 | 0.124 | 0.114 | 0.126 | 0.123 | 0.113 | 0.096 |
| **DAID** | 0.196 | 0.224 | 0.178 | 0.145 | 0.125 | 0.126 | 0.122 | 0.115 | 0.115 | 0.111 | 0.106 | 0.095 |
| **BIOA** | 0.136 | 0.144 | 0.139 | 0.128 | 0.129 | 0.125 | 0.118 | 0.114 | 0.114 | 0.112 | 0.108 | 0.093 |
| **TRI** | 0.098 | 0.115 | 0.12 | 0.124 | 0.11 | 0.112 | 0.109 | 0.106 | 0.11 | 0.109 | 0.105 | 0.091 |
| **DMSO** | 0.118 | 0.105 | 0.125 | 0.12 | 0.115 | 0.115 | 0.113 | 0.113 | 0.196 | 0.108 | 0.104 | 0.094 |
| **BPA** | 0.122 | 0.121 | 0.116 | 0.103 | 0.102 | 0.106 | 0.104 | 0.098 | 0.1 | 0.097 | 0.091 | 0.087 |

| Human &Cow | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **GEN** | 0.166 | 0.039 | 0.036 | 0.037 | 0.038 | 0.036 | 0.036 | 0.038 | 0.036 | 0.038 | 0.036 | 0.036 |
| **DAID** | 0.065 | 0.038 | 0.039 | 0.036 | 0.037 | 0.055 | 0.037 | 0.037 | 0.038 | 0.04 | 0.038 | 0.037 |
| **BIOA** | 0.077 | 0.039 | 0.037 | 0.037 | 0.037 | 0.038 | 0.036 | 0.036 | 0.036 | 0.038 | 0.038 | 0.038 |
| **TRI** | 0.041 | 0.045 | 0.041 | 0.039 | 0.038 | 0.037 | 0.039 | 0.036 | 0.037 | 0.039 | 0.037 | 0.038 |
| **GEN** | 0.512 | 0.587 | 0.571 | 0.618 | 0.615 | 0.585 | 0.573 | 0.512 | 0.521 | 0.289 | 0.159 | 0.117 |
| **DAID** | 0.498 | 0.493 | 0.544 | 0.543 | 0.516 | 0.34 | 0.198 | 0.146 | 0.11 | 0.12 | 0.131 | 0.114 |
| **BIOA** | 0.422 | 0.51 | 0.444 | 0.269 | 0.166 | 0.121 | 0.072 | 0.063 | 0.067 | 0.059 | 0.059 | 0.09 |
| **TRI** | 0.369 | 0.419 | 0.397 | 0.333 | 0.212 | 0.089 | 0.124 | 0.099 | 0.053 | 0.051 | 0.074 | 0.082 |

| PIG&RAT | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **GEN** | 0.42 | 0.508 | 0.506 | 0.482 | 0.569 | 0.478 | 0.609 | 0.557 | 0.471 | 0.296 | 0.178 | 0.105 |
| **DAID** | 0.603 | 0.533 | 0.566 | 0.566 | 0.551 | 0.427 | 0.23 | 0.157 | 0.115 | 0.109 | 0.104 | 0.102 |
| **BIOA** | 0.43 | 0.518 | 0.429 | 0.265 | 0.154 | 0.122 | 0.115 | 0.126 | 0.108 | 0.113 | 0.118 | 0.104 |
| **TRI** | 0.43 | 0.562 | 0.525 | 0.388 | 0.245 | 0.148 | 0.127 | 0.116 | 0.111 | 0.119 | 0.114 | 0.108 |
| **GEN** | 0.448 | 0.585 | 0.608 | 0.549 | 0.569 | 0.518 | 0.504 | 0.463 | 0.368 | 0.28 | 0.208 | 0.185 |
| **DAID** | 0.409 | 0.525 | 0.442 | 0.361 | 0.381 | 0.303 | 0.235 | 0.207 | 0.197 | 0.19 | 0.197 | 0.176 |
| **BIOA** | 0.31 | 0.341 | 0.281 | 0.217 | 0.225 | 0.212 | 0.221 | 0.208 | 0.187 | 0.2 | 0.197 | 0.19 |
| **TRI** | 0.275 | 0.272 | 0.244 | 0.198 | 0.199 | 0.196 | 0.194 | 0.189 | 0.178 | 0.194 | 0.181 | 0.175 |

| Sole&Zfish | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **GEN** | 0.936 | 0.911 | 0.858 | 0.854 | 0.727 | 0.791 | 0.961 | 0.746 | 0.805 | 0.647 | 0.797 | 0.705 |
| **DAID** | 0.82 | 0.855 | 0.743 | 0.746 | 0.767 | 0.886 | 0.852 | 0.789 | 0.8 | 0.87 | 0.809 | 0.77 |
| **BIOA** | 0.757 | 0.694 | 0.596 | 0.622 | 0.654 | 0.691 | 0.727 | 0.747 | 0.76 | 0.823 | 0.759 | 0.712 |
| **TRI** | 0.847 | 0.797 | 0.935 | 0.745 | 0.714 | 0.704 | 0.761 | 0.915 | 0.832 | 0.917 | 0.854 | 0.811 |
| **GEN** | 0.686 | 0.61 | 0.533 | 0.381 | 0.342 | 0.276 | 0.267 | 0.194 | 0.234 | 0.194 | 0.187 | 0.198 |
| **DAID** | 0.304 | 0.273 | 0.203 | 0.172 | 0.192 | 0.17 | 0.164 | 0.203 | 0.229 | 0.201 | 0.198 | 0.203 |
| **BIOA** | 0.176 | 0.221 | 0.162 | 0.167 | 0.145 | 0.143 | 0.181 | 0.17 | 0.158 | 0.179 | 0.197 | 0.227 |
| **TRI** | 0.155 | 0.154 | 0.139 | 0.116 | 0.129 | 0.11 | 0.116 | 0.153 | 0.201 | 0.229 | 0.216 | 0.155 |

## Appendix II

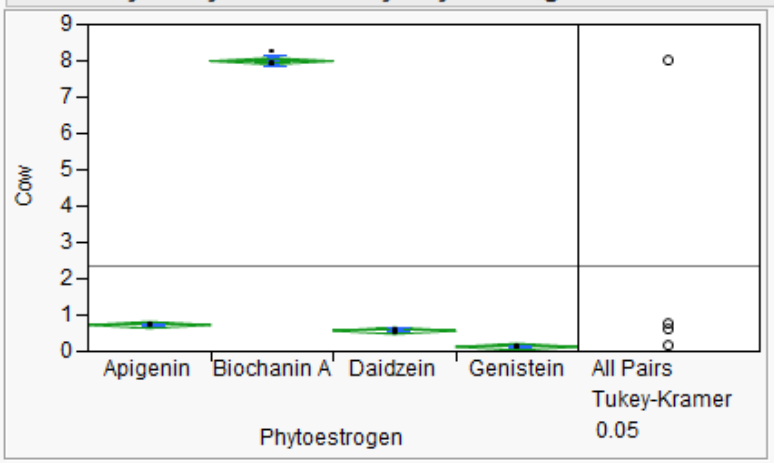
Calculated EC50 values

| | Phytoestrogen | Human EC50 (nM) | Porcine | Sole | Rat | Cow | Zebra Fish |
|---|---|---|---|---|---|---|---|
| 1 | Genistein | 0.085 | 0.11 | 0.194 | 0.086 | 0.094 | 4.723 |
| 2 | Genistein | 0.096 | 0.21 | 0.244 | 0.091 | 0.123 | 3.542 |
| 3 | Genistein | 0.084 | 0.29 | 0.188 | 0.085 | 0.167 | 2.781 |
| 4 | Genistein | 0.089 | 0.15 | 0.209 | 0.086 | 0.151 | 5.12 |
| 5 | Daidzein | 0.352 | 0.973 | 9.865 | 0.883 | 0.516 | 0.635 |
| 6 | Daidzein | 0.421 | 0.845 | 12.2 | 0.756 | 0.623 | 0.736 |
| 7 | Daidzein | 0.344 | 0.91 | 6.21 | 0.896 | 0.559 | 1.29 |
| 8 | Daidzein | 0.399 | 0.98 | 8.55 | 0.912 | 0.609 | 1.98 |
| 9 | Biochanin A | 8.01 | 7.91 | • | 14.6 | 7.92 | 1.3 |
| 10 | Biochanin A | 8.52 | 8.23 | • | 12.9 | 8.23 | 1.63 |
| 11 | Biochanin A | 8.96 | 8.92 | • | 13.4 | 7.89 | 1.06 |
| 12 | Biochanin A | 7.39 | 6.32 | • | 14.9 | 7.96 | 2.3 |
| 13 | Apigenin | 0.618 | 1.968 | • | 0.583 | 0.738 | • |
| 14 | Apigenin | 0.785 | 1.925 | • | 0.612 | 0.756 | • |
| 15 | Apigenin | 0.563 | 1.89 | • | 0.589 | 0.701 | • |
| 16 | Apigenin | 0.598 | 1.989 | • | 0.609 | 0.748 | • |

## Appendix III

Statistical Analysis on jmp 10 software

**Oneway Analysis of Cow By Phytoestrogen**




**Oneway Anova**

**Means and Std Deviations**

**Means Comparisons**

**Comparisons for all pairs using Tukey-Kramer HSD**

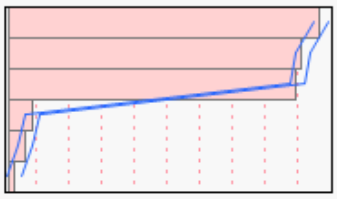
**Confidence Quantile**

**LSD Threshold Matrix**

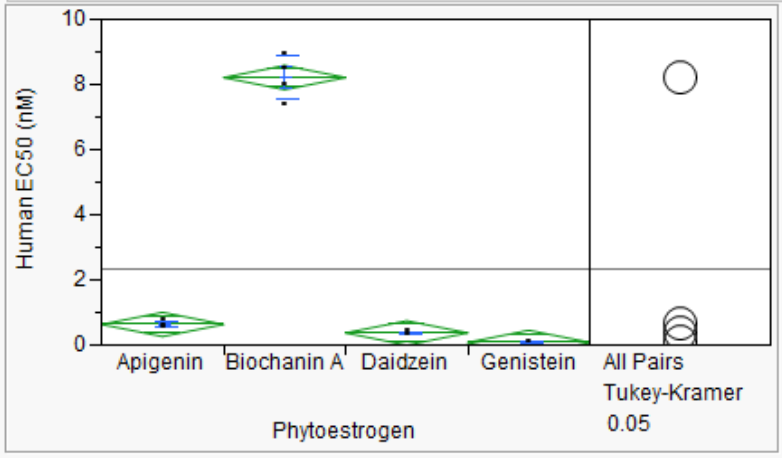
**Connecting Letters Report**

**Ordered Differences Report**

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| Biochanin A | Genistein | 7.866250 | 0.0595316 | 7.68951 | 8.042988 | <.0001* |
| Biochanin A | Daidzein | 7.423250 | 0.0595316 | 7.24651 | 7.599988 | <.0001* |
| Biochanin A | Apigenin | 7.264250 | 0.0595316 | 7.08751 | 7.440988 | <.0001* |
| Apigenin | Genistein | 0.602000 | 0.0595316 | 0.42526 | 0.778738 | <.0001* |
| Daidzein | Genistein | 0.443000 | 0.0595316 | 0.26626 | 0.619738 | <.0001* |
| Apigenin | Daidzein | 0.159000 | 0.0595316 | -0.01774 | 0.335738 | 0.0831 |



## Oneway Analysis of Human EC50 (nM) By Phytoestrogen




### Oneway Anova

### Means and Std Deviations

### Means Comparisons

#### Comparisons for all pairs using Tukey-Kramer HSD

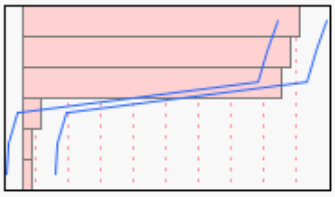
##### Confidence Quantile

##### LSD Threshold Matrix

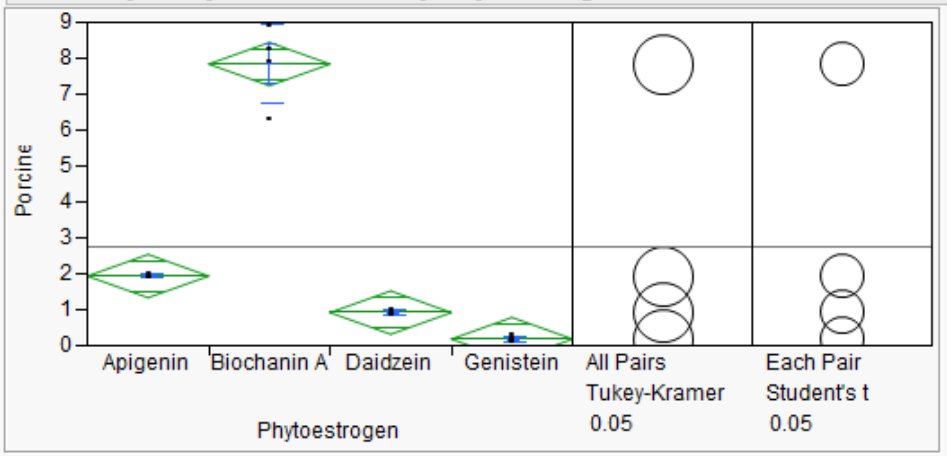
##### Connecting Letters Report

##### Ordered Differences Report

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| Biochanin A | Genistein | 8.131500 | 0.2418682 | 7.41344 | 8.849559 | <.0001* |
| Biochanin A | Daidzein | 7.841000 | 0.2418682 | 7.12294 | 8.559059 | <.0001* |
| Biochanin A | Apigenin | 7.579000 | 0.2418682 | 6.86094 | 8.297059 | <.0001* |
| Apigenin | Genistein | 0.552500 | 0.2418682 | -0.16556 | 1.270559 | 0.1564 |
| Daidzein | Genistein | 0.290500 | 0.2418682 | -0.42756 | 1.008559 | 0.6377 |
| Apigenin | Daidzein | 0.262000 | 0.2418682 | -0.45606 | 0.980059 | 0.7058 |



## Oneway Analysis of Porcine By Phytoestrogen




### Oneway Anova

### Means and Std Deviations

### Means Comparisons

#### Comparisons for all pairs using Tukey-Kramer HSD

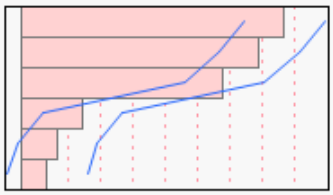
##### Confidence Quantile

##### LSD Threshold Matrix

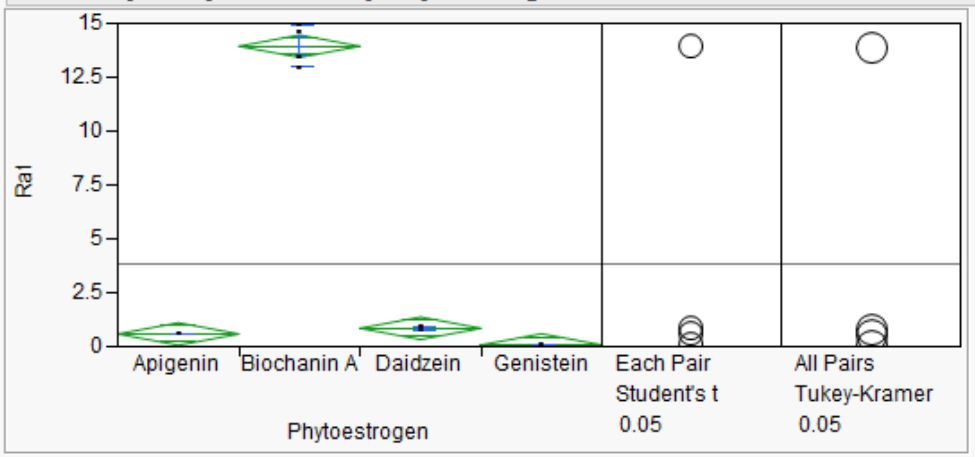
##### Connecting Letters Report

##### Ordered Differences Report

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| Biochanin A | Genistein | 7.655000 | 0.3910409 | 6.49408 | 8.815924 | <.0001* |
| Biochanin A | Daidzein | 6.918000 | 0.3910409 | 5.75708 | 8.078924 | <.0001* |
| Biochanin A | Apigenin | 5.902000 | 0.3910409 | 4.74108 | 7.062924 | <.0001* |
| Apigenin | Genistein | 1.753000 | 0.3910409 | 0.59208 | 2.913924 | 0.0036* |
| Apigenin | Daidzein | 1.016000 | 0.3910409 | -0.14492 | 2.176924 | 0.0939 |
| Daidzein | Genistein | 0.737000 | 0.3910409 | -0.42392 | 1.897924 | 0.2845 |



## Oneway Analysis of Rat By Phytoestrogen




### Oneway Anova

### Means and Std Deviations

### Means Comparisons

#### Comparisons for each pair using Student's t

#### Comparisons for all pairs using Tukey-Kramer HSD

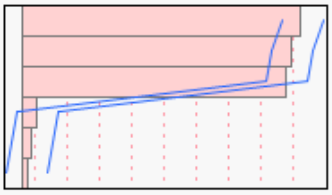
##### Confidence Quantile

##### LSD Threshold Matrix

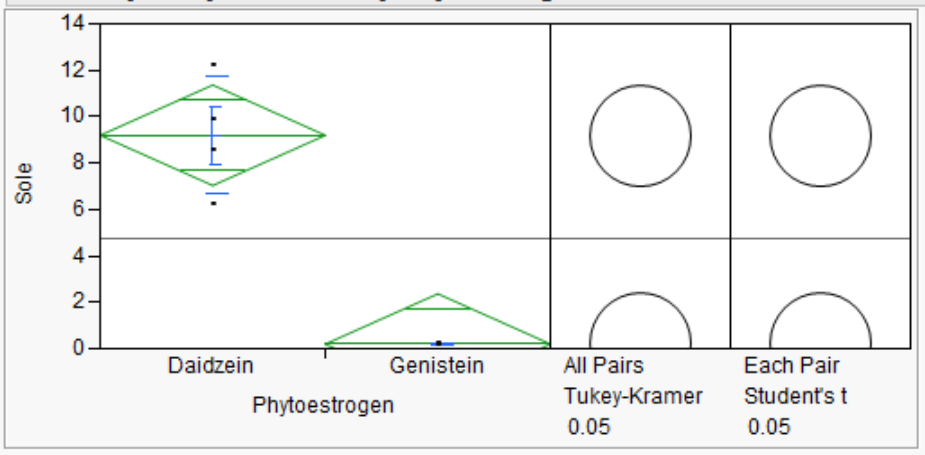
##### Connecting Letters Report

##### Ordered Differences Report

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| Biochanin A | Genistein | 13.86300 | 0.3382539 | 12.8588 | 14.86721 | <.0001* |
| Biochanin A | Apigenin | 13.35175 | 0.3382539 | 12.3475 | 14.35596 | <.0001* |
| Biochanin A | Daidzein | 13.08825 | 0.3382539 | 12.0840 | 14.09246 | <.0001* |
| Daidzein | Genistein | 0.77475 | 0.3382539 | -0.2295 | 1.77896 | 0.1549 |
| Apigenin | Genistein | 0.51125 | 0.3382539 | -0.4930 | 1.51546 | 0.4610 |
| Daidzein | Apigenin | 0.26350 | 0.3382539 | -0.7407 | 1.26771 | 0.8625 |



## Oneway Analysis of Sole By Phytoestrogen




Missing Rows 8

### Oneway Anova

### Means and Std Deviations

### Means Comparisons

#### Comparisons for all pairs using Tukey-Kramer HSD

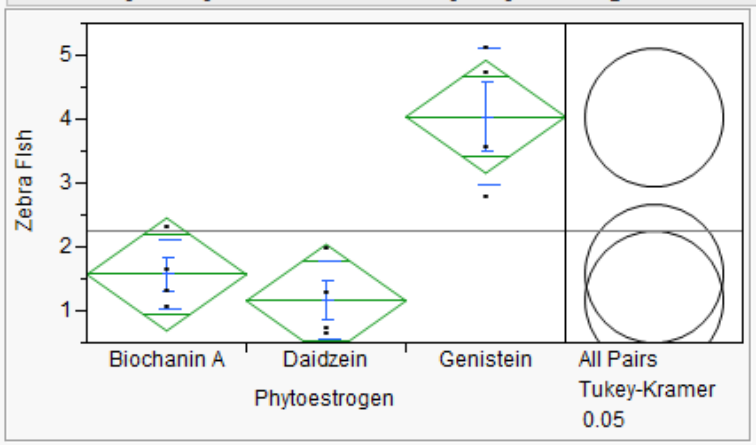
##### Confidence Quantile

##### LSD Threshold Matrix

##### Ordered Differences Report

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| Daidzein | Genistein | 8.997500 | 1.251884 | 5.934250 | 12.06075 | 0.0004* |

## Oneway Analysis of Zebra FIsh By Phytoestrogen




Missing Rows 4

### Oneway Anova

### Means and Std Deviations

### Means Comparisons

#### Comparisons for all pairs using Tukey-Kramer HSD

##### Confidence Quantile

##### LSD Threshold Matrix

##### Connecting Letters Report

##### Ordered Differences Report

| Level | - Level | Difference | Std Err Dif | Lower CL | Upper CL | p-Value |
|---|---|---|---|---|---|---|
| Genistein | Daidzein | 2.881250 | 0.5517719 | 1.34070 | 4.421803 | 0.0014* |
| Genistein | Biochanin A | 2.469000 | 0.5517719 | 0.92845 | 4.009553 | 0.0040* |
| Biochanin A | Daidzein | 0.412250 | 0.5517719 | -1.12830 | 1.952803 | 0.7428 |